CHEMICAL MODIFICATION AND CHARACTERISATION OF HORSERADISH PEROXIDASE AND ITS DERIVATIVES FOR USE IN ENVIRONMENTAL APPLICATIONS

By
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A thesis submitted for the degree of
Doctor of Philosopy

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Dublin City University January 1997

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 Philosopy, 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 own work.

Signed: Anne Marie O'Brien

Anne Marie O'Brien

Date: 14/2/97

ACKNOWLEDGEMENTS

I would like to thank most sincerely Dr. Ciaran O'Fagain for giving me the opportunity to carry out this research. His help and guidance over the past three and a half years is greatly appreciated.

I want to thank my husband Maurice who has been my inspiration and has put up with a lot over the years. He believed in me when I needed it most.

Thanks to all my friends who supported me specially Aideen, Fergal, Louise, Debbie, Deirdre, Geraldine, Celine, Teresa and Trisha.

To the SGO2 group, Ann, Damian and Sueann for being patient and understanding.

A big thank you to Brendan O'Connors research group who had a greater understanding of the mysterious workings of a computer than I. They are Oonagh, Damian, Sean, Ultan and Yvonne.

To the biology technicians, Monica, Roseanne, Teresa, Deirdre, Alison, Janice, Jeff, Ben, Brian, Robert and Trisha they have a lot to contend with.

Lastly and very importantly my family, Sheena, Pat, Sean and Vincent and specially my parents it would be impossible to have kinder, more understanding, more supportive or prouder parents.

ABBREVIATIONS

AA	Amino acid
A	Angstrom
ABTS	2,2'-azino-bis(3-ethylbenzthioline-6-sulfonic acid)
Abs	Absorbance
AADHA	Adipic acid dihydrazide agarose
AH_2	hydrogen acceptors
$AH^{\bullet}$	Free radical
Arg	Arginine
BCA	Bicinchoninic acid
BHA	Benzhydroxamic acid
BSA	Bovine serum albumin
CD	Circular dichroism
C6H5OH	Phenol
CO_2	Carbon dioxide
Cys	Cysteine
Da	Dalton
DH	Hydrogen donor
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DSC	Differential scanning clorimetry
DTT	Dl-dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EG-NHS	Ethylene glycol bis-succinic acid ester of N-hydroxysuccinimide
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
$Fe(CN_6)^{4-}$	Ferrocyanide
GdnCl	Guanidine hydrochloride
HCL	Hydrochloric acid
H_2O_2	Hydrogen peroxide
His	Histidine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
H_2SO_4	Sulphuric acid
I	Inactive state of protein
IPA	Indole 3-propionic acid
K	Equilibrium constant
kd	Dissociation constant
Km	Michaelis-Menten constant

k	Rate constant for protein inactivation
Lys	Lysine
M	Molar
mA	Milliamps
MOPS	3-[N-morpholino] propanesulfonic acid
Mr	Molecular weight
N	Native state of protein
NaCl	Sodium chloride
NaH_2PO4	Sodium dihydrogen orthophosphate
$Na2B_4O7$	Sodium borate
NaOH	Sodium hydroxide
$-NH_2$	Amino group
NH_3	Ammonia
NHS	N-hydroxysuccinimide
nm	Nanometer
NMR	Nuclear magnetic resonance
O_2	Oxygen
OPD	*o*-phenylenediamine
Ox	Oxidised form of enzyme
PA	Phthalic anhydride
PDA	Photo diode array
PEG	polyethylene glycol
Phe	Phenylalanine
RCA	Relative catalytic activity
Red	Reduced form of enzyme
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl) phosphine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TMB	3, 3', 5,5'-tetramethylbenzidine
TNBS	Trinitrobenzesulphonate
Tris HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Trp	Tryptophan
Tyr	Tyrosine
U	Unfolded form of enzyme
U.V./Vis	Ultraviolet/Visible spectroscopy
Ve	Elution volume
(v/v)	Volume per volume
Vmax	Maximum rate of enzyme reaction
(w/v)	Weight per volume
WT	Wild type

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ABSTRACT

The biochemistry and molecular properties of the widely-used horseradish peroxidase (HRP) are reviewed. The applications of HRP in diagnostics, sensors and waste treatment are also surveyed.

The research aims were to stabilise HRP by chemical modification to ascertain the characteristics of stabilised derivatives and to explore the capabilities of these derivatives in waste treatment applications. Assay methods for HRP activity were compared and thermoinactivation experiments were undertaken using a variety of conditions in order to devise workable, reproducible protocols for routine use. Systematic chemical modification of the available reactive HRP amino residues (Arg, Tyr and Lys) were performed. Arginine and tyrosine modified derivatives yielded little gain in stability Modification of lysine residues with cyclic anhydrides of aromatic carboxylic acids was undertaken. Of the compounds used, only phthalic anhydride yielded a thermally stabilised HRP (PA HRP). The overall stability characteristics of this derivative were investigated fully together with previously reported crosslinked HRP modifications. The tolerances of these two derivatives towards heat, organic solvents, hydrogen peroxide, pH and denaturants was enhanced. Both were immobilised on adipic acid dihydrazide-agarose (AADH-A). The stabilities of the immobilised, modified HRPs were compared with the modified-only counterparts. Unfolding of native and both modified HRPs were investigated using urea, guanidine chloride (GnCl), EDTA and heat. Unfolding over time was monitored using fluorescence and binding of the hydrophobic probe Nile Red. Results of the two methods agreed closely.

Attempts were made to fragment the HRP polypeptide so as to demonstrate the presence of crosslinks and, possibly, to identify the modified Lys residues. Unfortunately these experiments proved inconclusive. It was shown that recombinant HRP which, lacks the carbohydrate moiety, could withstand a chemical modification and be stabilised in a similar manner to the plant HRP.

A kinetic investigation of the HRP forms took place. Binding of the inhibitor BHA was found to be slightly less in the case of PA HRP. The pre-steady state and steady state kinetics of the HRPs using a variety of substrates were found to be in close agreement with each other. The significance of these results is discussed.

The use of both modified HRPs in waste treatment was explored. Modified HRP was as effective as native in the precipitation of phenols at 37 °C. Modified HRPs were more effective than the native at higher temperature where the native is inactivated. In the bleaching of dyes, modified HRPs were as effective as the native.

Thus, a new stabilised chemically modified form of HRP (PA HRP) has been described and the capabilities of this and the EG HRP in two distinct types of wastewater treatment have been characterised.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Biochemistry and Distribution of HRP

Peroxidases are a class of enzyme that are distributed widely throughout the animal and plant kingdoms. Their widespread presence would suggest that they are an essential component of nearly all living systems. They play an active role in metabolism, participating in coupled oxidations and protecting cells against peroxide poisoning. They are also known to be involved in cell wall biosynthesis. In plant cells they are located mainly in the cell wall, vacuoles, transport organelles and on membrane bound ribosomes (Gasper et al., 1982). Peroxidases occur naturally in mammals and have been discovered in such diverse areas as human saliva, adrenal medulla, liver, kidney and leucocytes (Pruitt et al., 1990). It has also been shown that a major component of the soluble protein of human cervical mucus is a type of peroxidase, (Shindler et al., 1976).

Peroxidases are one of the most extensively studied groups of enzymes and the literature is full of reviews and basic research papers dating back to early this century. The extracellular peroxidases, particularly Horseradish peroxidase isoenzyme C (HRP C) helped to usher in the modern era of enzymology (See Dunford et al., 1991 for a recent review). They have been characterised into three classes based on sequence alignments and biological origin (Welinder, 1992).

1) Class I: the intracellular peroxidase; including Yeast cytochrome C peroxidase (CCP) and a number of plant and bacterial peroxidases found in cytosol or chloroplasts.

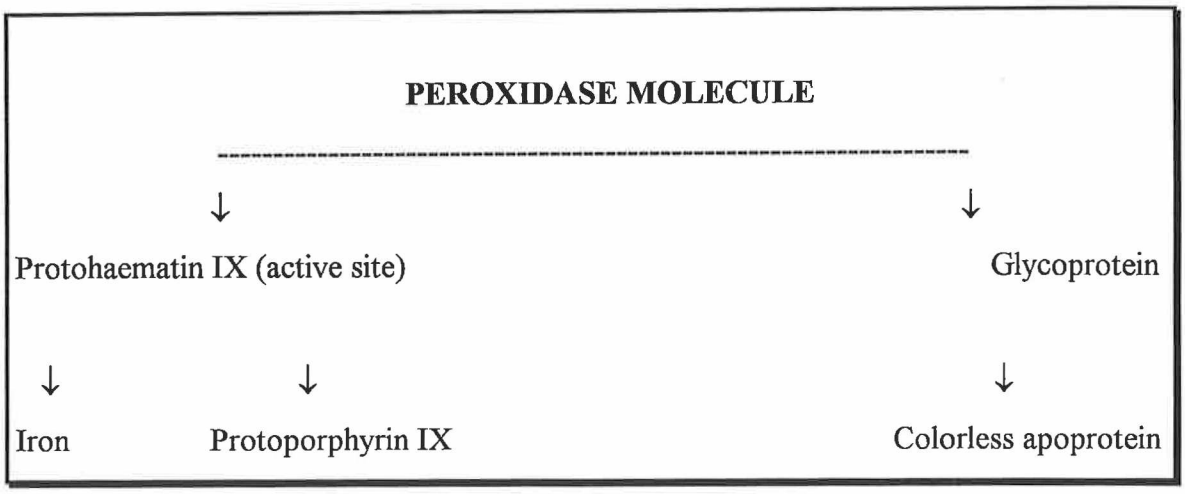
2) Class II: secretory plant peroxidases; These are monomeric glycoproteins with four conserved disulphide bridges and two conserved calcium sites.

3) Class III: secretory plant peroxidases; also monomeric glycoproteins with four conserved disulphide bridges and two conserved calcium ions, however, location of the disulphides differs from class II (Schuller et. al., 1996). HRP C is a class III peroxidase

1.1.1 Structure of the HRP molecule

Horseradish peroxidase is an oxidoreductase (donor; hydrogen peroxide oxidoreductase; E.C. 1.11.1.7; HRP). Like all peroxidases, it functions in the transfer of hydrogen peroxide from hydrogen donors (DH) (Tijssen, 1985). As many as 40 isoenzymes have been described, some of which may be HRP with variations in carbohydrate composition. HRP is divided into at least six groups of isoenzymes. The acidic HRP-A, the neutral and slightly basic HRP B and C, the highly basic peroxidases HRP D and E (Shannon et. al., 1966), and a neutral peroxidase, HRP-n (Bartonek-Roxa et al., 1991). The three most studied isoenzymes of the above group are HRP C, HRP A and the highly basic peroxidases. Native HRP consists of a polypeptide chain containing 308 amino acid residues (Welinder, 1979). The amino terminus is blocked by a pyrlolidine 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 co-ordinated 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 haem and a lysine molecule in the apoprotein. HRP isoenzyme C consists of two compact domains, between which the haem group is positioned (Welinder, 1979). The iron group has six co-ordinated positions, four of which are occupied by porphyrin nitrogen atoms and the fifth by a protein group, histidine, (Haschke and Friedhoff, 1978). The sixth position can be occupied by various compounds. Peroxidases appear to operate through the exchange of substrate in this position. The components of the peroxidase molecule have been described by Welinder (1979) (Fig. 1.1)

Figure 1.1 Various Components of Peroxidase Molecule



The covalent structure of HRP consists of two domains, one of which incorporates the haem group. Eight neutral carbohydrate chains are attached through asparagine residues (Welinder, 1979). The carbohydrate residues are mainly located in the C-terminal half of the polypeptide. There are four disulphide bridges located between the cysteine residues. No free thiol groups exist and only two titratable histidines occur. The carbohydrate portion of the protein by which approximately 18% of the molecular weight is accounted for (Ohlsson et. al.,1977) appears to shield the six lysines on the protein backbone. The enzyme contains a single tryptophan residue that can be fluorimetrically detected, but is not located at the active site (Paul, 1963).

HRP is a metalloprotein and calcium plays an important role maintaining the structural stability of the enzyme (Smith et al., 1990). The protein contains two moles of calcium per mole of enzyme. Treatment of HRP with 6 M guanidine hydrochloride-10 mM EDTA for approximately 10 hours can remove the bound calcium, resulting in a significant decrease in thermal stability. Addition of calcium can restore the stability. Recombinant HRP C will not refold in the absence of calcium (Smith et. al., 1990), in fact calcium has been reported to be an essential constituent of HRP. The molecular weight of HRP C is 44kDa including the carbohydrate moiety. The working pH range of HRP is 4.0-8.0 (Paul, 1963).

1.1.2 Catalytic Cycle of HRP C

As stated previously HRP C is one of the most widely studied peroxidases, and because of this, the catalytic cycle has been well characterised (Fig. 1.2). Peroxidases catalyse the oxidation of substrates. This process is characterised by the formation of Compound I and Compound II, which are active intermediates. The initial step of the reaction involves the two electron oxidation of the haem group. On addition of hydrogen peroxide the resting enzyme undergoes a two electron ($2e^-$) oxidation to give a high oxidation state intermediate called Compound I an active unstable intermediate. The iron of Compound I is in the ferryl state (FeIV=O) and is magnetically coupled to a porphyrin π-radical cation (Smith et. al., 1992). This intermediate can react 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 decomposes slowly. Suitable electron donors, employed in the formation of Compound II, would include most chromophores used in the peroxidase activity assays. A reductive step returns Compound II to the resting state (Smith et. al., 1993). An excess of hydrogen peroxide results in total enzyme inactivation, and reaction between Compound II and hydrogen peroxide results in the formation of Compound III which is relatively inert and not thought to be an essential part of the peroxidase cycle (Smith et. al., 1992)

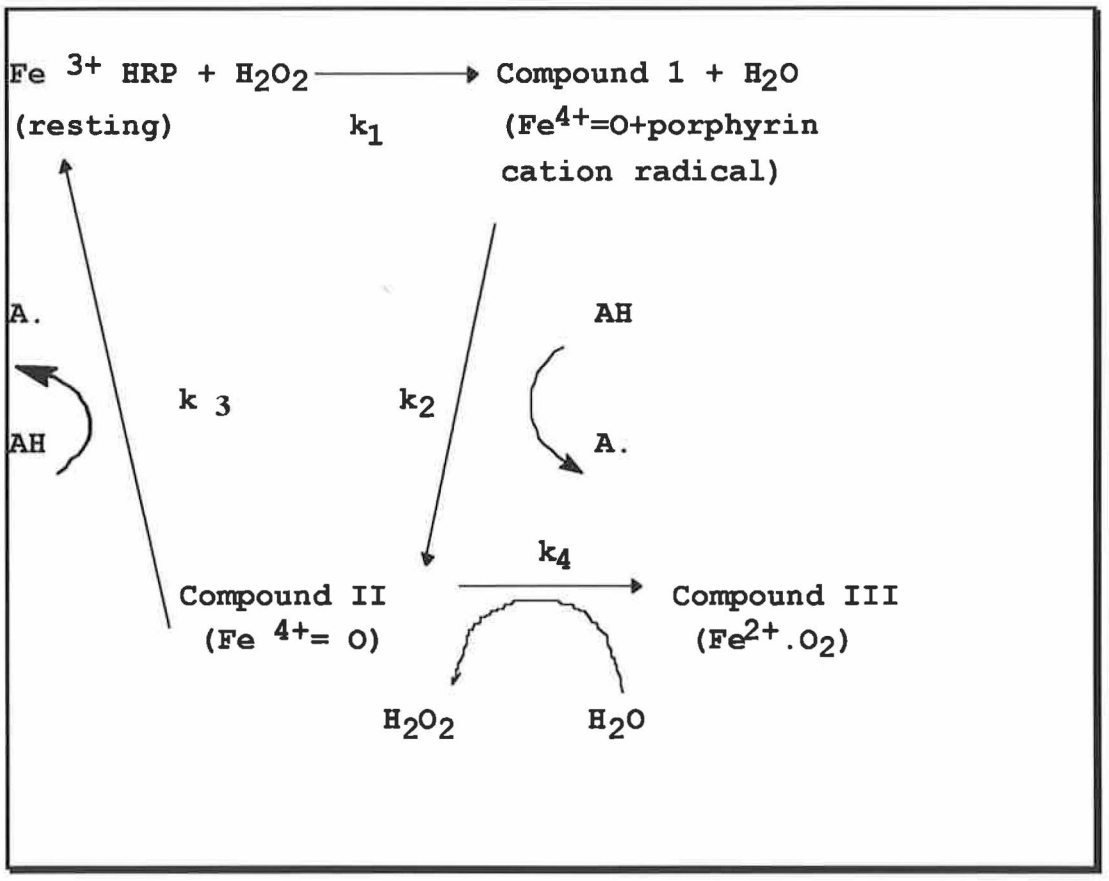
1.1.3 Peroxidase Activity Assays

Peroxidases are capable of catalysing the oxidation of a wide range of substrates. Basically, HRP decomposes 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 (Conyers et al., 1991). The native HRP is regenerated by the hydrogen donor, which is oxidised.

A wide range of indicator molecules or substrates exist and these can be incorporated into a number of assays used to detect HRP activity. Some of the chromogenic substrates used include TMB, ABTS and MBTH. A number of other

substrates such as o-phenylenediamine (OPD) were commonly used, but the oxidation products were found to be carcinogenic and, therefore, their use was discontinued.

Figure 1.2 A Generalised Scheme for the HRP Reaction Pathway



1.1.4 Stability of HRP

Native HRP exhibits characteristics similar to that of an ideal enzyme. Its catalytic activity can be maintained for long periods of time at room temperature. Activity may also be maintained in buffers of varying ionic strength and in the pH range 4 and 11 for short intervals of time, even though its optimum pH is between 6 and 8 (Paul et al., 1963).

Peroxidase has demonstrated the ability to function while suspended in water miscible organic solvents, even when there is a minimal amount of water bound to the surface (Klibanov, 1989). It is not unusual for enzymes to function in anhydrous organic solvents such as hexane or toluene (Ryu and Dordick, 1992), however, protein inactivation is often the result of incubation in water miscible solvents. 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 (Gorman and Dordick, 1992). Water is involved in all non-covalent interactions that help to maintain protein conformation. It has been suggested that enzymes such as HRP need approximately 10^3 molecules of water per enzyme molecule. This layer of water is thought to act as a lubricant, with the ability to form hydrogen bonds with various functional groups.

The situation with water-miscible organic solvents is, however, different. Gorman and Dordick, (1992) described the desorption of tritrated water bound to HRP in a range of organic solvents such as methanol or hexane. Desorption of tritrated water was almost immediate, with most of the desorbable T_2O released in the first five minutes. This phenomenon is known as "water stripping" (Zaks et al., 1988a) and appears to account for the catalytic sensitivity of nearly all enzymes in water-miscible organic media. Zaks et al. (1988b) reported that a number of fundamental properties of enzymes could be deduced by suspension in organic solvents. The thermostabilisation of HRP and other proteins have been extensively reviewed in recent times (Gianfreda and Scarfi, 1991). The role of calcium is vital in maintaining the structural stability of HRP. In spite of the fact that calcium does not participate in the peroxidase catalytic reaction, the thermal stability of the enzyme is significantly decreased when exposed to guanidine hydrochloride made up in EDTA, removing the calcium from HRP, (Haschke and Friedhoff, 1978). The role of calcium is discussed in more detail at a later stage.

1.1.5 Protein Stabilisation

Enzymes can be stabilised by a number of methods, including immobilisation, chemical modification, use of additives and protein engineering. A number of very good reviews have been published on the above techniques. It is not

within the scope of this present review to discuss in detail all of the methods of enzyme stabilisation therefore the interested reader is referred to Ryan et al. (1994). There are enzymes in existence that possess relatively high stability. These may occur in organisms that are capable of living at elevated temperatures (55-100 °C) (Thermophiles). Such enzymes often display relatively high activity in the presence of protein denaturants, for example guanidine hydrochloride (GnCl), urea, detergents organic solvents and proteolytic enzymes. It would therefore, be of considerable interest to examine these enzymes in relation to mesophilic enzymes.

1.2 Recombinant Enzymes

The advances in genetic engineering over the past number of years have made it possible to manipulate foreign genes for expression in micro-organisms. Perhaps the main achievement of the techniques developed for isolating, characterising, sequencing and manipulating genes, has been the development of "protein engineering". This has allowed the cloning and expression in micro-organisms of genes coding for rare and valuable proteins which can then be altered and produced on an industrial scale (Scragg. 1988). An intimate knowledge of a protein structure and function is required in order to engineer proteins so that they can be distributed, stored, administered and used in a variety of applications, including the design of new therapeutic compounds (Hol, 1987) and the production of enzyme biocatalysts suitable for use in industrial non-aqueous media. It will soon be possible to produce multifunctional enzymes with a number of catalytic domains grafted onto sections of stable protein (Ringe, 1989). Engineered proteins are of use in the fine chemical, pharmaceutical, toxic waste and food industries. Proteases are used for the conversion of protein into peptides and amino acids for food, detergents, cheese production and pharmaceuticals, invertase for conversion of sucrose into invert sugar for confectionery, glucose oxidase for conversion of glucose into gluconic acid for food and drinks, fungal, plant and bacterial enzymes (peroxidase, polyphenol oxidase and hydrolase, etc.) are used for toxic waste conversion (James and Crabbe, 1990).

The technology for introducing defined mutations at specific sites has been available for a number of years and a number of enzymes have been used as models to

indicate how specific mutations affect enzyme stability, specificity, substrate binding and catalysis. The methodology is essentially as follows:

1) To define the required change in function,

2) To clone the gene for the protein so that it can subsequently be mutated and expressed,

3) To obtain knowledge of the protein structure,

4) To propose specific residues which could be altered to achieve the desired functional change and to model the consequences of such changes,

5) To effect promising mutations based on the modelling studies,

6) To express and process the mutant proteins and

7) To determine the functionality of the new protein (James and Crabbe, 1990).

See Fig. 1.3 for a schematic of strategies used for protein engineering.

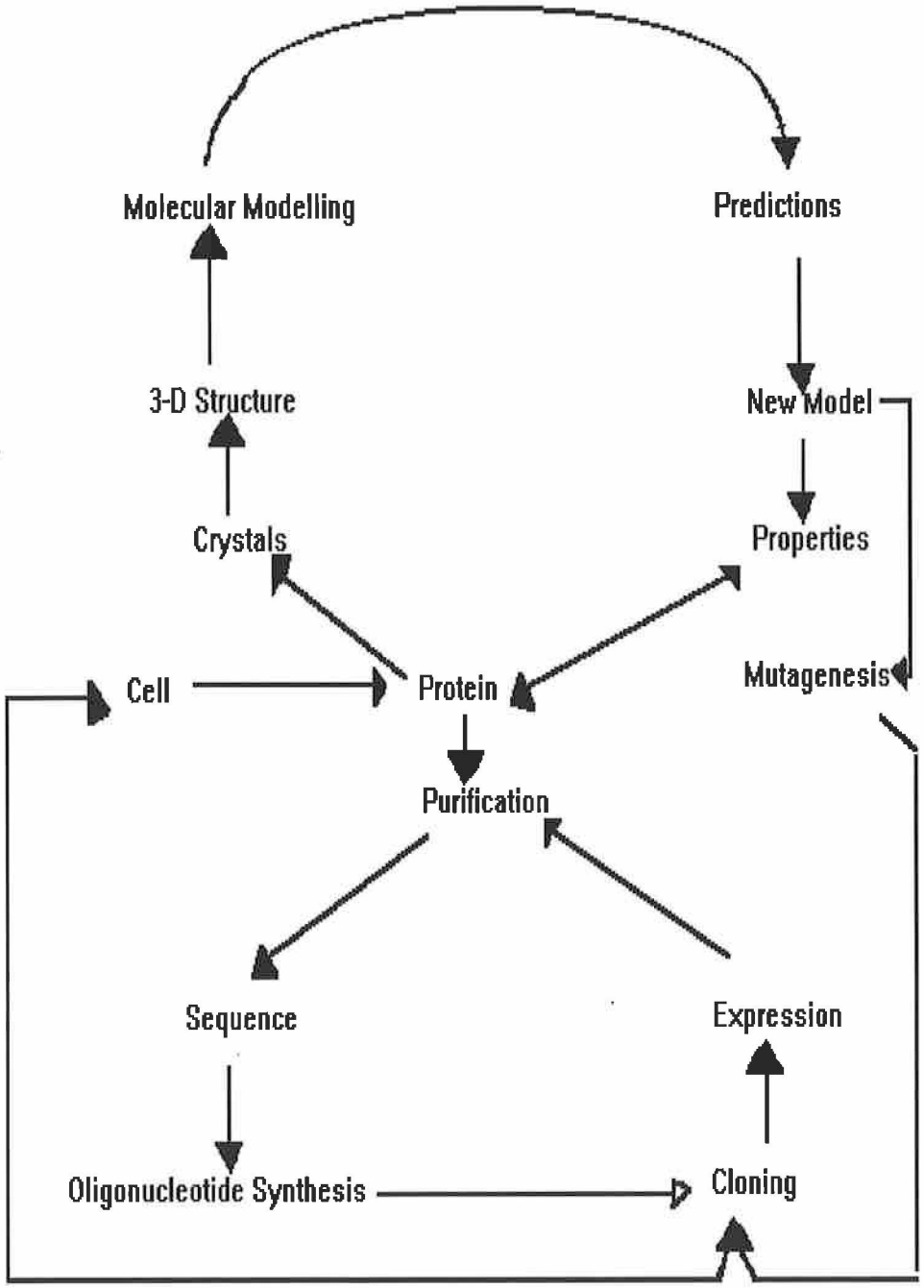


Fig. 1.3 **Diagrammatic scheme for obtaining protein structure and function information and for protein engineering**

1.2.1 Techniques

There are a number of techniques involved in genetic manipulation to alter the coding sequence of a cloned gene. The mutations may be insertions, deletions and base-substitutions. The changes can be random or site-specific.

1) Insertion or deletion mutations:

Excision or insertion of defined DNA sequences in a gene, involving enzymatic methods to cleave the DNA, to remove or insert a DNA sequence or to ligate the ends.

2) Base-substitutions:

(a) fragmented mutagenesis, where an entire fragment is exposed to a mutagen (Hong and Ames, 1971).

(b) Site-mutagenesis:

1) Incorporation of nucleotide analogues: modified bases can be incorporated to produce a mixture of products or chemical methods can be used, (Shortle et al., 1978).

2) Chemical methods: the predominant mutagenic reaction is in the formation of a Schiff's base resulting in a derivative exhibiting ambiguous base pairing properties

3) Oligonucleotide-directed mutagenesis:

a) An oligonucleotide that is partly complementary to the nucleotide sequence in the target DNA is synthesised.

1.2.2 Construction of Synthetic Gene Encoding Recombinant HRP (HRP C*)

On the basis of the protein sequence for HRP C published by Welinder (1979), a synthetic gene, 940 nucleotides in size, has been designed and constructed using codons commonly found in highly expressed *E. coli* and mammalian cells (Smith et al., 1990. and Ortlepp et al., 1989). The plasmid pSA233, without the *tac* promoter and the plasmids pSA261 and pSA262 (with the *tac* promoter) were used by Egorov et al. (1992). They found that pSA233 was replicated, however, without expression. Whereas using the pSA261 plasmid there was expression but there was no excretion of the recombinant protein observed. Egorov et al., (1992) used only the pSA261 transformants to express the recombinant protein. Smith et al., (1990) also noted that it was necessary to reclone the HRP gene into a *tac* expression vector, resulting in the HRP gene being expressed at high levels. Translation was presumed to be initiated at the engineered AUC, encoding methionine at the N-terminus of the protein. The cDNA coding for HRPn (a neutral peroxidase) was found to be growth-inhibiting and toxic upon expression in *E. coli.* The expressed product demonstrated no activity. These effects were thought to be mediated by the produced polypeptide since no inhibition was observed when the cDNA was ligated to the vectors in the wrong reading

frame or reversed orientation. The above sequence was found not to contain the proximal Histidine.

1.2.3 Inclusion bodies

The formation of insoluble refractile protein aggregates (inclusion bodies) was found to be the only way to ensure that *E. coli* survived the production of HRP C*. Bartonek et al. (1994) suggested that *E. coli* was not a very suitable system for HRP C* production in large quantities. This is despite the fact that Smith et al. (1990) and Ortlepp et al.(1989) had successfully regenerated active HRP produced in *E. coli.* In fact, Smith is of the opinion that expression in *E. coli* or in the baculovirus systems are among the best available methods. The workers mentioned have cloned and expressed HRP C* which accumulates as inactive, insoluble aggregates inside the cell. No toxic effect of the cDNA sequence was reported. It was reported however, that there were extensions at both the N and C-termini, although neither extension is required for activity. It is possible that C-terminally extended HRP C may represent an inactive pro-enzyme awaiting proteolytic cleavage to activate it (Smith et al., 1990). This is in contrast to the C-terminally blocked active HRP C produced by Hartmann et al. (1992).

Using the procedure of Smith et al. (1990) above Lignin peroxidase (isoenzyme H8) designated LipP*) was successfully over-expressed by Doyle and Smith, (1996) as insoluble aggregates contained in inclusion bodies of *E. coli*. Based on these and (colleagues) previous results they postulated that this type of procedure may be suitable for the recovery of most, if not all active recombinant peroxidases.

1.8.2 Selection of *E. coli* Strain

The strain of *E. coli* used has been shown to have significant effects on expression levels of HRP. *E. coli* JM 109/pSA 261 showed high levels of enzyme expression (30% of the total protein in the cell pellet) with the strains HB 101 and TG 1 having expression levels lower than 10 % and only 2% when *E. Coli* DIM 103 was used (Egorov et al., 1992). See table 1.1 for an outline of the expression of recombinant HRP in different systems, and Table 1.2 gives a brief outline of expression of various peroxidases in *E. coli*

Table 1.1 **Expression of Recombinant HRP**

Expression System	comment	Ref, Year
E. coli	1) Strain of *E. coli* affects levels of expression, 2-30% 2) Plasmids without *tac* promoter replicated without expression	Egorov et al., 1992
E. coli	1) Over expression of haem 2)10-20% of total cell protein expressed as HRP	Edwards, 1989
E. coli	1) Unglycosylated 2) 20-fold increase in the amount of protein synthesised when C-terminal 72 amino acids deleted	Ortlepp et al., 1989
E. coli	1) Low temperature expression in the presence of haem; however, low expression (2%) and low activity 2) Reverted to reactivation from inclusion bodies	Egorov et al., 1994
E. coli	1) Isolated, cloned and characterised 3 cDNA 2) No expression	Fujiyama et al., 1988
E. coli	1) Low yield (2-3%), non-glycosylated 2) Extended N-terminal	Smith et al., 1990 & 1992
Mammalian cells	1) Significant levels of activity detected	Edwards, 1988
Saccharo-myces cerevisiae	1) Hyperglycosylated 2) C-terminal polypeptide extensions present	Vlamis-Gardikas et al., 1992
Baculovirus	1) Enzyme terminally blocked 2) Good yields 3) Active site of recombinant and native identical	Hartmann & DeMontellano, 1992

Table 1.2 **Expression of other Recombinant Peroxidase, in *E. coli***

Peroxidase expressed	comment	Ref , Year
CcP	Expression 100% more efficient than in Yeast	Fishel et al., 1987
LiP	Low yield of active enzyme(1%)	Doyle & Smith, 1996
BSP	Expression and activity, protohaem correctly incorporated into expression product	Lopersert et al., 1989

CcP = Cytochrome c Peroxidase, LiP = Lignin Peroxidase, BSP = *Bacillus stearothermophillus* Peroxidase, HRP = Horseradish peroxidase

1.2.5 Expression of Horseradish Peroxidase in *E coli*

HRP is divided into at least six groups of iso-enzyme, the acidic HRP-A, the neutral and slightly basic peroxidases, HRP-B, -C, the highly basic peroxidases, HRP-D, -E, (Shannon et al., 1966), and a neutral peroxidase, HRP-n (Bartonek-Roxa et al., 1991) identified from cDNA sequence. The different carbohydrate groups of the various isoenzymes are generally thought to have different physiological roles in plants.

Producing active recombinant HRP facilitates the production of the enzyme both in its homogeneous form and in large amounts, which is important for crystallisation (Morita et al., 1991) and for detailed studies on the reaction mechanisms of HRP, also providing opportunity to carry out point mutations making it more suitable for industrial and clinical purposes.

When constructing a recombinant gene the ideal situation is to have a particular cleavage site between the fusion protein and the target recombinant protein, which is not present in the sequence of the recombinant, otherwise the recombinant product will also be cleaved.

1.2.6 Folding of Recombinant HRP

1.2.6.1 Folding Mixture

A problem frequently encountered in the expression of heterologous proteins in *E. coli* is that the gene product generally appears as in an insoluble, inactive form contained

within inclusion bodies (Smith et al., 1990; Egorov et al., 1991 & Edwards et al., 1988). Insoluble inclusion bodies can be easily sedimented into the outer membrane fraction on low speed centrifugation (10,000*g*,15 min). After partial purification, the HRP C* readily dissolves in 8M urea. The reduced Apo-HRP C* is incubated in a folding mixture to re-fold the HRP C* and produce active enzyme. The folding mixture is generally the same for all the recombinant HRP C produced. Smith et al., (1990) included Tris, 6M urea, $CaCl_2$, bovine haem, EDTA, and oxidised glutathione. Egorov, (1991) included 2% mercaptoethanol in the mixture. Edwards et al., (1988) noted that the insoluble aggregated protein within the cell, had a pink coloration. This they explained as an over expression of haem in the protein. This was not encountered by the other workers who reported the production of Apo-enzyme. Edwards et al. (1988) had no reason therefore, to incorporate haem into the folding mixture.

1.2.6.2 Incorporation of calcium:

When calcium is absent from the refolding mixture, reactivation does not proceed at all, indicating the importance of disulphide bridge formation. HRP contains four intramolecular disulphide bonds. These are essential for maintaining the stability of the HRP molecule. (Egorov et al., 1992; Edwards et al., 1988 & Smith et al., 1990). However, disulphide bonds are difficult to form in the reducing bacterial environment. The role of calcium ions in the folding procedure emphasises their importance in the structure of peroxidase. The implication of the work is that the binding of calcium ions is an obligatory step in the folding pathway that must occur before correct disulphide bridge formation and haem incorporation can be completed. This is certainly true for the unglycosylated recombinant enzyme at least. Calcium ions are known to have important structural roles in many proteins. However, to date peroxidases are the only haem protein in which they have been reported as a constituent.

1.2.7 Role of Glycosylation

1.2.7.1 Glycosylation of HRP Expressed in Baculovirus

Horseradish peroxidase is highly glycosylated, approximately 18% of its molecular weight (44,000 Da) being accounted for by carbohydrate residues attached to eight glycosylation sites (Welinder, 1979a). This, however complicates its expression as bacteria do

not glycosylate proteins. A number of workers have succeeded in expressing active recombinant HRP in *E. coli* (See Table 1.1). The active protein was obtained unglycosylated and in small amounts in active form and was refolded as described earlier. Hartmann et al. (1992) reported the expression and overproduction of HRP C* in insect tissue culture using a baculovirus transfer vector. The molecular weight of the recombinant protein was almost 43,000 Da. The blocked N-terminus is consistent with the fact that the N-terminus is blocked in HRP isolated from horseradish root (Welinder, 1979). (The baculovirus is able to N-terminally block proteins because chloramphenicol acetyltransferase and tyrosine hydrolase are N-terminally blocked when expressed in baculovirus). The purified protein was also sur-gylcosylated. Both mammalian and insect cells initially transfer high-mannose oligosaccharide precursors to asparagine residues in the polypeptide chain. However, where mammalian cells add a variety of terminal sugars to a core oligosaccharide after trimming the high mannose structure, insect cells do not. The difference in the N-linked oligosaccharide structure did not alter the catalytic activity function with respect to that of the native HRP.

1.2.7.2 Glycosylation

The role of glycosylation in a number of recombinant proteins has been studied. (Tsuda et al., 1990 and Higuchi et al., 1992). In the recombinant HRPs produced in *E. coli* the lack of glycosylation did not appear to affect the catalytic activity of the HRP. It has been demonstrated that native plant HRP deglycosylated by chemical or enzymatic means has the same initial activity as the native enzyme, but is quickly inactivated, suggesting that oligosaccharides play an important role in plant peroxidase physiology (intracellular transport and localisation) and may prevent enzyme inactivation by active radicals of substrates, (Tams and Welinder, 1990). Thermoinactivation experiments were carried out on HRP C* by Egorov et al., (1994) and its activity at 56 °C over time was compared with that of the naive plant HRP. The HRP C* was found to be three times less stable than the native HRP, even though they had similar catalytic properties. This decrease in thermal stability according to Egorov et al, (1994), was due to the absence of oligosaccharide chains protecting the enzyme against an attack of the substrates radicals. Tams and Welinder, (1995), found that HRP was still active even after all of the carbohydrate except GlcNAc was removed. Bonnaffe et al., (1993) also showed that the catalytic activity of HRP was not affected on the removal of sugar-chains, however, they reported that the thermal stability was decreased.

removal of sugar-chains, however, they reported that the thermal stability was decreased. They also reported that fixing sugars on to the enzyme increased its thermal stability by a factor of 5-6, therefore it would appear that glycans do not interfere with substrate access and substrate binding in HRP.

Studies were carried out on the role of carbohydrate in recombinant human erythropoietin (expressed in baby hamster kidney cells). It was found that N-linked sugar chains are not required for *in vitro* activity, however, *in vivo* activity was lost. This indicates that the presence of N-linked sugar is required for the hormone to reach the target sites, (Tsuda et al., 1989 and Higuchi et al., 1992). Wingfield et al., (1988), compared the conformation and stability of recombinant-derived human protein from *E. coli,* mammalian cells by urea-gradient electrophoresis. They showed that for human GM-CSF the presence of covalently attached carbohydrates does not significantly change either protein stability, or the kinetics of unfolding and refolding. It would appear that there is some ambiguity still as to the role of carbohydrate in the stability of proteins.

1.2.8 Aromatic Substrates

1.2.8.1 Benzhydroxamic acid (BHA)

The true substrates of plant peroxidases are poorly defined, although auxin and lignin precursors, such as ferulic acid may be involved. There is a very broad range of aromatic molecules that interact with plant peroxidases, and most of the available information has come from studying HRP C.

HRP forms complexes readily with BHA, an aromatic hydroxamic acid used in studies of the aromatic donor molecule binding site of plant peroxidases (see Fig. 7.1 for the structure of BHA). While there is no known physiological role for BHA, there is a defence mechanism in the *Graminae* against fungi, bacteria and insects for the related compound 4-hydroxy-1, 4-benzoxazin-3-one (Niemeyer, 1988). The complex of BHA and HRP C is of interest, as it exhibits two distinctive features which set it apart from complexes formed with other aromatic donor molecules (Schonbaum, 1973):

1) The formation of complex with resting state HRP C. This results in perturbation of the properties of the haem iron atom, with spectrophotometric, EPR and magnetic susceptibility measurements indicating the generation of a fully high-spin haem.

co-ordinate high-spin resting state to a six-co-ordinate high spin state, with a water molecule acting as the sixth ligand (Smulevich et al., 1991)

2) There is a small K_d value when compared with other typical aromatic donors. BHA binds with values of 2.4 μM (Smith et al., 1992) while the latter have millimolar values (Paul & Ohlsson, 1978).

These differences have been ascribed to polyfunctional hydrogen-bonding interactions with the protein. (Schonbaum, 1973). NMR spectroscopy has proved to be a most useful technique in developing a detailed description of this peroxidase complex, despite the difficulties inherent in studying a high molecular mass system which is also paramagnetic, (Veitch & Williams, 1995). Many key resonances are perturbed on BHA binding and this has been demonstrated by one-dimensional NMR experiments. Veitch & Williams (1995) have confirmed that the binding site of BHA must lie towards the distal side of the haem plane. This was confirmed recently by Schuller et al. (1996).

The binding modes for BHA appear to be unique to HRP C, for example :

a) HRP A2 in its resting state binds BHA much more weakly than does HRP C,

b) BHA is in slow exchange between resting state HRP C bound and free forms and there is essentially a fast regime in operation in the case of HRP A2. (Veitch & Williams 1995a). There are subtle variations among the amino acid residues in the vicinity of the haem group between different isoenzymes (Welinder, 1992) and these may play a determining role in the binding of aromatic donor molecules.

1.2.9 The Aromatic Donor Molecule Binding Site

A number of research groups are in the process of determining an X-ray structure for HRP. However, there has of yet been no high resolution crystal structure of HRP available, although the X-ray structure of HRP C has been solved by Smith et al., (1995) based on the structure of the recently solved Peanut peroxidase. The structure of HRP C has in the past been compared to Cytochrome C peroxidases (CCP). Welinder et al., (1992) demonstrated the homology between the two enzymes. This sequence homology between CCP and HRP has implicated similar residues in the active site for HRP as for CCP. ^{1}H NMR studies have, in the absence of a high resolution crystal structure of HRP, provided direct support for the existence of a co-ordinated His with

extensive imidazolate character. The His42 and Arg38 have also been found to have dispositions relative to the haem very similar to that of CCP. (Thanabal et al., 1988).

Much less is known about the aromatic substrate binding pocket in HRP and, because Cytochrome C is the substrate for CCP, its sequence homology with HRP is not useful. The native substrates for HRP include phenols and aromatic amines. Recently, though, it had been suggested that the structure of HRP may be in fact closer to that of Peanut peroxidase (PNP). The sequence homology of PNP to HRP C is 50%, higher than that of any other peroxidase of known structure, with many of the substitutions being conservative. Certainly it appears that the overall fold of HRP will be identical to that of PNP. The sequences align well with only three insertions and a C-terminal extension of two or three residues for HRP C. All three insertions, as well as the C-terminal extension, are on the molecular surface and all are sufficiently far (> 15 Å) from the haem iron, so as not to effect any significant changes in the functionality of HRP C as compared with PNP (Schuller, et al., 1996)

1.2.10 Substrate Access Channel

The identification of the binding site(s) for aromatic donor molecules has been hampered due to the lack of crystallography data. However, model building on the basis of CCP structure has suggested that the core of CCP and HRP may be conserved. (Smith et al., 1995 and Loew et al., 1995). NMR data, (Veitch & Williams, 1995, Veitch et al., 1995a), enzyme inactivation studies, (Gilfoyle et al., 1996, Harris et al., 1993) and the use of hydrogen donor substrates (e.g. guaiacol, p-cresol and benzhydroxamic acid (BHA), (Roderiguez-Lopez & Smith, 1996, Smith et al., 1992, Gilfoyle et al., 1996), have resulted in the location of the aromatic substrate binding site near the delta-meso-haem edge which react in a general way at these sites. It has long been anticipated that peroxidases, especially the class III peroxidases, will have an aromatic binding site in this region. The exact position of the binding sites have not been unambiguously located yet. The distal pocket opening of PNP is, in fact, surrounded by more hydrophobic and uncharged residues than that of CCP, particularly on the left. The side chain of two phenylalanine residues (Phe) have been implicated in the binding procedure (Roderiguez-Lopez et al., 1996). It has been noted that the benzhydroxamic acids interact very strongly with the haem edge at methyl C18H3, while the methylbenzyhydroxamic acids

interact mainly with the distal His42 and the Phe A side chains (Veitch & Williams, 1995). His 42 participates directly with the binding site but the Phe involvement is indirect. The resting and cyanide-ligated states of HRP incorporating an amino acid substitution, binds BHA 3-4 times more weakly than the respective wild type enzyme and substitution of Phe for Val at position 41 has a substantial effect on k_i, decreasing it eight-fold (Smith et al., 1992). As it does not participate directly in the binding site, this difference must be accounted for by a subtle change that occurs in the aromatic donor molecule binding site as a result of this substitution (Veitch et al., 1995a). Amino acid sequence comparison and NMR comparison of a number of plant peroxidases have suggested that the assignment of these Phe resonances is to Phe 142 and Phe 143 (Phe A and Phe B). Sakurada et al., (1990). However, the substrate access channel of PNP, like that of other known peroxidase structures, seems to lack any clearly distinguished pocket which would constitute a specific binding site for aromatic substrates. Schuller et al., (1996), therefore, raised the question, do Peroxidases form true enzyme-substrate complexes prior to substrate oxidation?. In fact, they say that such a complex could be disadvantageous because the formation of a tightly bound substrate radical after oxidation may lead to enzyme inactivation.

1.2.11 His 42 and Arg 38

A common feature of all peroxidases is the strong conservation of the amino acids in the region of the two histidine residues, located on either side of the haem binding pocket (See Fig. 1.3). The proximal histidine 170 provides an imidazole ligand that is covalently bound to the haem iron. While the distal histidine 42 is not co-ordinated to the iron, it is thought to play a major role as a general acid base catalyst, facilitating peroxide anion binding to the haem and subsequent cleavage of the dioxygen bond, (Gilfoyle et al., 1996 & Roderigeuz-Lopez & Smith, 1996). The high resolution X-ray structure of Bakers yeast Cytochrome C Peroxidase of Paulos et al. (1980) led to the proposal of the above acid base catalysed mechanism. The resulting intermediate contains an ionised peroxide molecule (HO_2^-) covalently bound to the iron atom, with the proton from the α-oxygen being transferred to the distal histidine. The positively charged guanidium group of Arg38 shifts towards the bound anionic peroxide ligand (Smith et al., 1993). In the CCP variants R48K and R48L (R38 in HRP C) have been characterised

kinetically and shown to react with hydrogen peroxide to form Compound I up to 200 times slower than for the wild type native CCP.

Roderiguez-Lopez & Smith, (1996) showed that the mutation of His42 and Arg38 residues in the HRP C resulted in the enzyme binding to BHA 10^3 and 5×10^3 times less tightly, respectively, than either the glycosylated or HRP C*. Therefore, either mutation greatly weakens the BHA binding. This is consistent with a model of HRP C where the positive charge of Arg38 would interact electrostatically with the partial negative charge which develops on the oxygen side of BHA. In the case of His42 a hydrogen bond is formed with its imidazole group. Both residues react therefore in a manner similar to that which is thought to occur during Compound I formation.

Gilfoyle et al., (1996), dramatically illustrated how distal catalytic residue mutations ([R38K] HRP C* i.e. Arginine replaced by Lysine and [His42L] HRP C* i.e. Histidine replaced by leucine), destroyed the high affinity binding site for BHA. They demonstrated that HRP C* bound so tightly during chromatography on a benzhydroxamic-acid-sepharose affinity matrix that 0.5 M sodium borate was required to disrupt the protein/ligand complex. However, the distal pocket mutant [R38K] HRP C* was not retained at all by the affinity column. The second order rate constant for Compound I formation was also found to be decreased by as much as 1,200 - fold, Roderiguez-Lopez et al., (1996a). Additional evidence that Arg38 is directly involved in the binding of aromatic substrates was presented by Roderiguez-Lopez et al., (1996a), when they showed that the dissociation constant for the binding of guaicol and p-cresol to the ferric enzyme increased when Leucine was substituted for Arg38. He suggested that modifications of the hydrogen bond interactions in the complexes formed when they bound to the enzyme and that this could possibly be the reason. Which is consistent with the effect on the binding of these two substrates to Compound I and II (i.e. an increase in the K_m values). A local perturbation is not sufficient to explain these large differences in affinity, which result from a single-site substitution.

Binding studies where HRP was modified with a number of arginine specific reagents indicated that the HRP modified with 2,3-butanedione or glyoxal (arginine specific) could not bind Guiacol at all and that TNM modified (tyrosine reagent) bound it but with reduced affinity. Both modified enzymes were able to form

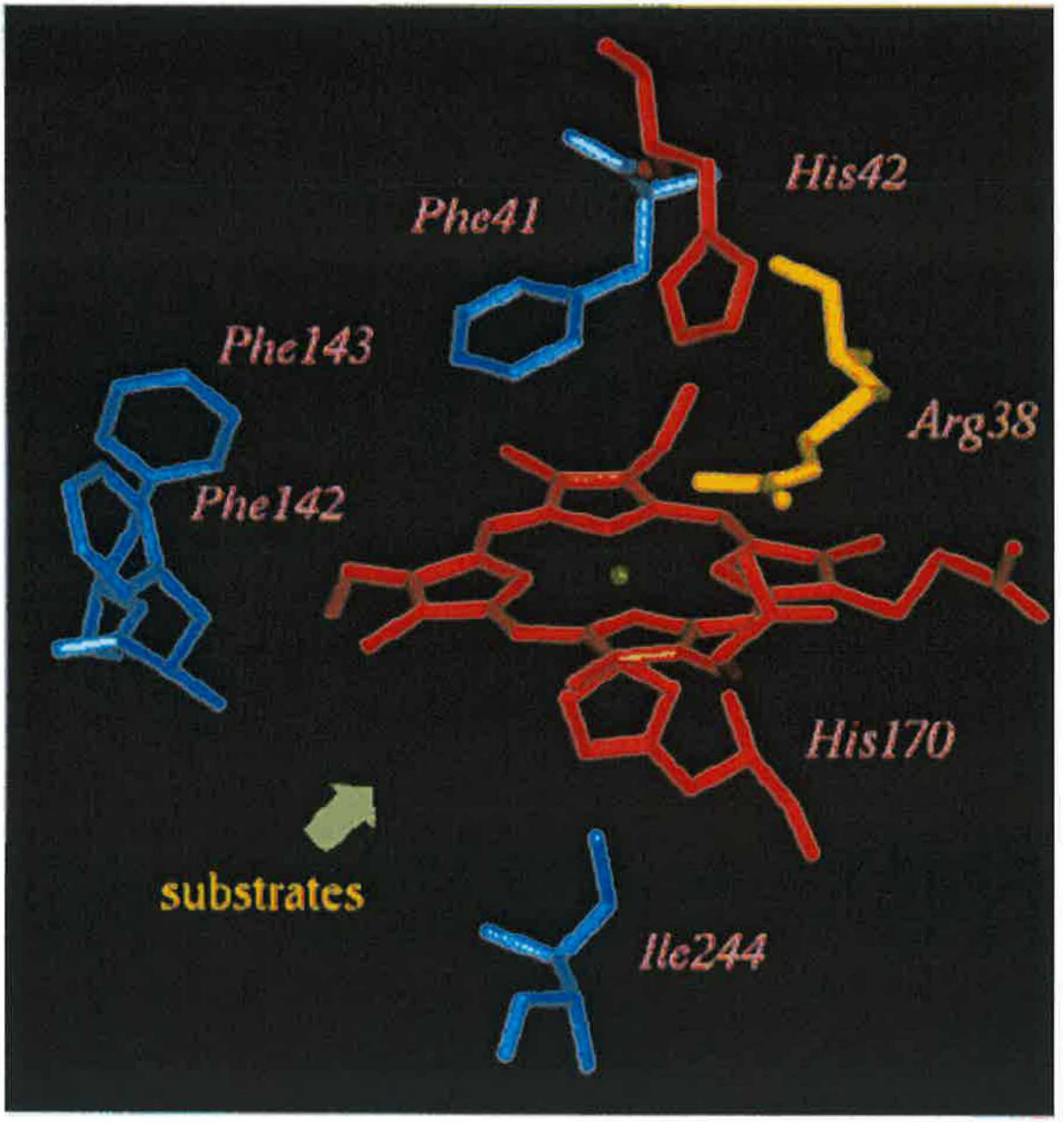


Fig. 13. Energy optimised homology model of the haem cavity of HRP C. (Roderigeuz-Lopez et al., 1996a)

Compound II with H_2O_2, but reduction of Compound II to native HRP with Guiacol did not occur in the arginine modified enzyme (possibly due to lack of binding) (Adak et al., 1996). In 1979 Riordan noted that arginine residues in a protein will often serve as an anionic site for the binding of a negatively charged group in a substrate or co-factor. For this reason direct electrostatic interactions between the positively charged guanidium group of Arg38 and the partial negative charge developed on the oxygen of the phenolic group of substrates, such as guiacol and p-cresol, could be the simplest explanation. This would cause the substrate to be orientated in the active site in a manner similar to that proposed for the binding of BHA (Smith et al.,1993). It appears that the main role of Arg38 is to make the formation of Compound I an irreversible reaction. However, Arg38 has other roles, as the conservative substitution of this site renders Compound II undetectable and modifies the properties of the aromatic donor binding site. A mutation which disrupts the haem-linked hydrogen bonding network will abolish the binding of BHA (Veitch et al., 1992). A table of various site-mutations carried out on recombinant HRP C and the effect of these mutations can be seen below (Table 1.3-1.5).

Table 1.3 Effect of Arginine 38 Site-Mutations on HRP C*

Mutation	Comment	Ref,
Arg38→Leu [R38L]HRP C*	1) fast recombination of carbon monoxide	Meunier et al., 1995
	2) reduced form does not bind cyanide	
	3) increased sensitivity to H_2O_2	Roderiguez-Lopez & Smith 1996
	4) BHA binding weakened	
	5) involved in cleavage of O-O bond of peroxide	Roderiguez-Lopez et al., 1996a
	6) Compound I more stable	
	7) increased K_m for guaiacol, p-cresol and ABTS	
Arg38→Lys [R38K] HRP C*	1) reduced activity with ABTS as substrate	Hiner et al., 1994 and 1995
	2) increased sensitivity to H_2O_2	
	3) alkaline transition occurs when pH is raised from 6.6 to 8.6	Sanders, 1994
	4) increase in both 6c high (HS) and low spin (LS) haems	Smulevich et al., 1994
	5) distal mutation does not bind BHA	Veitch et al., 1994
	6)the two proximal His170 protons are shifted	

Table 1.4 **Effect of Histidine Site-Mutations on HRP**

Mutation	Comment	Ref.
His 42→Leu [H42L] HRP C*	1) recombination of CO faster than WT native HRP C 2) reduced form does not bind cyanide 3) rate constant for reaction with H_2O_2 decreased 4) BHA binding 1,000 times weaker than for WT native HRP C	Meunier et al., 1995 Roderiguez-Lopez & Smith, 1996
His 42→Arg [H42R] HRP C*	1) recombination of CO faster than for WT HRP C 2) reduced form does not bind cyanide	Meunier et al., 1995
His 170→Gln [H170Q]HRP C*	Stable Compound I formation	Venables & Smith, 1994

WT = wild type enzyme,

HRP = Horseradish peroxidase,

BHA = Benzhydroxamic acid,

Table 1.5 Effect of Phenylalanine Site-Mutations on HRP C

Mutation	Comment	Ref.
Phe 142→Ala [F142A] HRP C*	1) structural integrity of aromatic donor binding site is altered 2)Cyanide-ligated form binds BHA weakly	Veitch et al. 1995
Phe 41→Leu [F41L] HRP C*	1) oxidation of alkyl acryl sulphides is faster than for WT native HRP C	Ozaki et al., 1994
Phe 41→Thr [F41T] HRP C*	1) oxidises thioanisole and p-chlorothioanisole more rapidly but with lower enantioselectivity	Ozaki et al., 1994
Phe 41→Trp [F41W] HRP C*	1) does not bind BHA	Smulevich, 1994
Phe 41→Val [F41V] HRP C*	1) decreased reactivity towards H_2O_2 (k_1) 2) k_2 and k_3 constants with para-aminobenzoic acid 2.5 and 1.5 times slower than WT nat HRP C 4) decreased activity with ABTS	Smith et al., 1992
	5) increased affinity for BHA	Smith et al., 1992 & Veitch et al., 1992
	6) increase in 6-cHS form at expense of 5-cHS form	Smulevich et al., 1994
	7) haem methyl resonance of C8 is shifted	Veitch et al., 1992
Phe143→Ala [F143A]HRP C*	1) increased activity with ABTS as substrate 2) identical binding constants for BHA as for WT nat HRP C 3) Likely to be located at the periphery of the haem access channel 4) 2 fold increase in sensitivity to H_2O_2	Hiner et al., 1995 Veitch et al., 1995 Hiner et al., 1994

1.3 Use of Peroxidase in Wastewater treatment

1.3.1 Introduction

The treatment of biological waste is by no means a new idea. It is a well established technology which has produced many beneficial products and contributed towards conservation through recycling of substrates for various final uses. Although water covers 80% of the earth's surface, 90% of this water is unavailable for human consumption as it is contained in the oceans (another 2% is in the polar ice caps). Pollution has been described 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 interference with legitimate uses of the environment" (McEldowney et al. 1993). The type of pollutants vary and they may be metals, a range of organic compounds or gases. Their relative importance in terms of pollution is linked to their perceived toxicity to humans. It is important therefore, that there are effective waste treatment systems, especially in highly industrialised and populated areas. The problem has increased in the past number of years due, in part, to intensive farming, the growth of the agro-industry and urban centres. In 1976 the U.S. Resource Conservation and Recovery Act (RCRA) attempted to address the proper management of hazardous waste by identifying the origins of such waste, developing appropriate technologies for waste management and the implementation of regulations to protect public health and the environment in an effort to gain a response from both the public and industry (Pirages, 1988). The EU has developed similar strategies, through its Community Action Program on the environment. The main objectives of the programme are a) to reduce the quantity of non-recoverable waste, b) to recover and reuse waste for raw materials and energy purposes, c) management and safe disposal of non-reusable waste (Ferranti, 1987). A lot of progress has been made in the last number of years; however, there is always room for improvement. Toxic waste can now be traced from its point of origin to the place of final disposal. Under recent amendments to the 1976 RCR act, the U.S. Environmental Protection Agency (EPA) is required to evaluate and if necessary, prohibit the disposal of certain wastes. The EU has a "black list" for its most dangerous toxic compounds and a "grey list" for the less toxic (Mason, 1991). In the same way, the U.S. EPA has a list of 129 chemicals that are classified as priority pollutants. The list includes 11 extractable

organic compounds, including phenol. Ingestion of small amounts of phenol can cause nausea, vomiting, paralysis and even death from respiratory failure or cardiac arrest. Such compounds can also bioaccumulate and so affect the food chain (Keith & Telliard, 1979). Phenol and its derivatives are detectable in drinking water at very low levels. A phenol taste can arise at phenol concentrations below 1μg L^{-1}, pure phenols however cannot be tasted in drinking water at 1μg L^{-1}; levels (Bewley et al., 1991).

1.3.2 General Peroxidase Catalysed Reactions

Peroxidase is capable of catalysing the oxidation of a wide variety of compounds. Due to the strong redox properties of its oxidised form and also the long distance electron transfer processes that can occur in proteins. The size of the substrate does not appear to be an important factor, with substrates ranging in size from phenols to biopolymers such as lignin (Sawahata & Neal, 1982). Dordick et al. (1986a) reported that HRP is capable of depolymerising lignin in an organic medium such as dioxane but not in aqueous solution. The rate of peroxidase-catalysed breakdown of lignin was shown to be much lower under anaerobic conditions, unlike classical peroxidative coupling of phenol in water which is unaffected by molecular O_2. It has been reported that peroxidase is the only enzyme known to be capable of polymerising *p*-coumaryl alcohol, a lignin precursor, into lignin-like polymers in the presence of hydrogen peroxide (Harkin & Obst, 1973). 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 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 (Klibanov et al. 1984).

Until recently, very few studies had 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 with the cytotoxic agen 9-methoxyellipticine. The near-complete conversion of the substrate is observed with peroxidase in the reaction mixture. The decrease in the quinone-imine

concentration during the conversion is related to a co-polymerisation of the quinone-imine and the starting material. It was also noted that methanol is formed during such reactions (Meuiner and Meuiner, 1985). Gillette et al. (1982) reported the first N-demethylation type reaction involving peroxidase. It involved the catalysis of aminopyrine (4-dimethyl-aminoantipyrine) in the presence of peroxidase. Its oxidative capacity with *o*-phenylenediamine (OPD) resulted in the development of an activity assay which is sensitive to 16ng L^{-1} of peroxidase and is linear in the 16-200ng L^{-1} range (Bovaird et al., 1982).

1.3.3 Peroxidase Catalysed Polymerisation of Phenols and Aromatic Amines

Phenol-formaldehyde resins are among the most practically important groups of phenolic polymers. They have numerous applications and are used in areas such as wood composite, fibre bonding, laminates, foundry resins, abrasives, friction moulding, materials coating, adhesives and flame retardants. (Brode, 1982). Phenol resins like Novolak and resol are very important materials in the industrial field (Uyama et al., 1994). These oligomers are formed by the condensation of formaldehyde with either phenol itself or alkyl (aryl) substituted phenols, in particular p-cresol, p-tert-butylphenol, p-phenylphenol (Dordick et al., 1986b)

In recent years the synthesis of such phenol-formaldehyde resins has been looked into closely due to the various toxic effects of formaldehyde. The formaldehyde reaction is based on high temperatures, with undesirable side reactions, leading to poor control of polymer structure and molecular weight (Akkara et al., 1992). For these reasons, there has been a lot of study into alternative technologies to produce these polymers. Inorganic, e.g. copper-based, catalysts are used to manufacture poly(phenylene oxide) resins, however, their use is limited to phenols having substituents in both *ortho* positions (Braun & De Long, 1982). Therefore, the enzymatic polymerisation of phenols is being explored in greater detail. Enzymes are useful catalysts for organic synthesis. Due to their capabilities in catalysing reactions at phenomenal rates and their environmentally non-toxic properties, they have been used for the synthesis of small molecules, such as L-amino acids and aspartame. Large

molecules such as cellulose and polypeptides have also been prepared (Derango et al., 1992). Horseradish peroxidase catalyses the oxidative coupling of a variety of substrates including phenols and aromatic amines through activation by hydrogen peroxide (Westerfield & Lowe,1942).

1.3.3.1 Reaction in Organic Media

In aqueous media, the reaction is not very effective due to poor substrate solubility, and polymerisation does not proceed further than low molecular weight oligomer formation before precipitation limits further chain growth. Therefore, it is better to carry out the reaction in organic media in order to sustain the growing chain in solution. (Akkara et al., 1992). The ability of HRP to function in a variety of organic solvents and its ability to polymerise phenols in such media has been reported (Klibanov, 1986). Peroxidase-catalysed polymerisation of phenols and anilines resulted in the formation of polymers with molecular weights between 1,400 and 2,000 Da (Dordick et al, 1986b), which are commercially useful. Kobayashi (1994) reported the production of a bioactive polymer, which produced potentially antimicrobial compounds. The polymerisation reaction is also feasible in a reversed miceller system (i.e. the enzyme activity is maintained within water-in-oil microemulsion) with yields of up to 95% polymer. This compares well to other systems such as 85% dioxane (Akkara et al., 1992). Polymers have been successfully produced by a number of researchers using HRP in organic media. These include: Dordick (1987), Uyama et al. (1994), Derango et al., (1992). Also see Dordick (1992) for a review on enzymatic and chemoenzymatic approaches to polymer synthesis.

Ryu and Dordick (1990) noted that, while polymers in excess of 25,000 Da could be prepared in a variety of water-miscible solvents using HRP as catalyst, that the enzyme was also capable of depolymerising lignin and coals, making it of interest to the coal bioprocessing industries. The ability to polymerise phenols is also of tremendous interest in the treatment of waste water contaminated with phenols.

1.3.4 Treatment of Phenolic Waste

In the field of industrial waste pollution, the treatment of phenolic material contained in industrial waste water is of primary concern. There are several

methods available including recovery, incineration, adsorption, biological treatment and chemical methods. All of these methods, although feasible and useful, suffer from one or many drawbacks, such as high cost, incomplete purification, formation of hazardous by-products or low efficiency, (Pokora, 1991). A number of these methods will be discussed briefly.

1.3.4.1 Microbial Treatments

Under controlled conditions, micro-organisms suitable for treating or removing phenols from waste can be developed. Biological treatment of such compounds is feasible over a wide concentration range. Three bacterial species have been reported to be capable of degrading phenol i.e. *Klebsiella pneumoniae, Serratia liquefaciens* and *Pseudomonas putida*. It was reported that commercial preparations of these species were capable of similar behaviour (Lewandowski et al., 1988).

In order to obtain high initial biomass concentration and fast reaction times, immobilised microbial cells are often used. These immobilised cells have the advantage of repeated use if the system is properly designed (Flint, 1987). Extended half lives for *Streptomyces fradiae* activity have been reported by Kokubu et al. (1981) when optimal conditions are employed. Drawbacks of the microbial system include slow diffusion of substrate (phenol) through the cell wall, cell lysis and adverse cell reactions yielding products that may interfere with the main reaction. The use of a microbial system, however, avoids the expense of enzyme purification and the enzymes are arranged in a logical order inside the cell. It is very hard to reproduce this artificially with enzymes in a reactor system (Kokubu et al., 1981).

1.3.4.2 Recovery of Phenols

This system can be applied to wastewaters from coal refining plants and chemical works. It uses benzene as the extraction solvent in a packed column with a counterflow. A multistage solvent extractor can also be employed, which uses isopropyl ether as the solvent. The boiling point of this solvent is lower than that of phenol and the solvent can, therefore, be distilled from the phenol for further use. Recoveries of up to 99.7% have been reported. Additional treatments may be required for significant amounts of phenol remaining in the waste stream however (Wurm, 1981).

1.3.4.3 Incineration

Incineration processes burn materials in sludges and produce inert ash which maybe disposed of. By-products of phenol incineration include water and carbon dioxide according to the following reaction,

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

Phenol has poor solubility in water ($\cong$ 10%); therefore, mechanical mixing is required. It is necessary to add a supplementary fuel to less-concentrated phenol mixtures to maintain the combustion temperatures (871 °C) (Lanouette, 1977).

1.3.4.4 Activated Carbon

This treatment is useful for treating low concentrations of phenolic waste. It is quite a versatile system and will remove other aromatic, non-polar organic materials from the waste stream (Hager & Rizzo, 1974). The final phenolic concentration in the treated effluent can be as low as 1 mg L^{-1} or less. Overloading of the activated carbon can be a problem. Also, the phenolic waste may need to be pre-treated to remove suspended solids prior to the activated carbon stage. A relatively low energy consumption technique has been developed where supercritical fluid CO_2 passes through the loaded activated carbon and dissolves the adsorbed material thus, removing it. This has proved particularly useful in the case of pesticides, as they can be resold as dry pesticides (U.S. EPA report, 1980). Lanouette, (1977) has devised a testing system to determine if activated carbon methodologies are applicable to particular waste-treatment problems.

1.3.4.5 Chemical Oxidation Methods

There are a number of reasons why chemical oxidation methods may be applied to phenol destruction. (1) Treatment of batches that are high in phenolic content and low in other organics, is less expensive than incineration or biological methods. (2) pre-treatment before biological treatment ensures the introduction of uniform waste levels into aeration basins and (3) chemical oxidation can act as a final step after other

treatment methods (Lanouette, 1977). Oxidising agents such as chlorine dioxide, ozone, potassium permanganate ($KMnO_4$) and hydrogen peroxide have been applied to convert phenol to less harmful substances. Oxidising agents have been used in water treatment to remove iron and manganese and to reduce tastes and odours. Hydrogen peroxide is an effective oxidiser of phenol over a wide concentration range in the presence of iron salts. The reaction is independent of temperature and, fortunately, peroxide appears to exhibit no adverse effects on the environment (Lanouette, 1977).

The use of ozone in the treatment of waste water started in the 1970s in America. Its main use has been in the treatment of coal-conversion waste. The end products are carbon dioxide and water, and ozone has been shown to be more effective than hydrogen peroxide in the destruction of phenols. The potency of ozone is unaffected by pH; however, a major disadvantage is that it decomposes quickly to molecular oxygen. It can be employed in either a batch or continuous treatment system, but due to its instability, it must be generated on site. (This can be achieved by passing dry air through an electrical charge Sandstrom et al., 1979.)

Chlorine dioxide attacks the benzene ring of the phenols to form compounds which are odourless and tasteless. It oxidises chlorophenols formed during chlorination converting them to inoffensive compounds. Chlorine dioxide is generated at the treatment site, either from chlorine gas, or from hypochlorite. Potassium permanganate used for paint stripping wastes, also has the capability of oxidising nine times its weight of phenol. The oxidation of phenol is carried out according to the following scheme and works best in an alkaline environment (Lanouette, 1977).

$$3C_6H_5OH + 28KMnO_4 + 5H_2O \rightarrow 18CO_2 + 28KOH + 28MnO_2$$

The manganese dioxide sludge which results from the precipitation reaction must be removed.

1.3.4.6 Aeration

Phenols can be removed to some extent by aeration. Typical aeration methods could be classified as (1) diffused, (2) submerged turbine and (3) surface. The reactors need to be designed so that there is a sufficient air supply to satisfy the demands of the technique. The liquid depth of the reactor is usually about 5 meters. The air

requirements for the reactor are based on mass transfer coefficients for the particular device.

1.3.5 Phenol Removal from Aqueous Solution using Immobilised Peroxidase

The use of immobilisation techniques for enzymes is not a new concept and a number of immobilisation methods are currently available. These are discussed in Section 4.7. The most important features of immobilisation are low cost of the solid support, the production of reusable material and the elimination of waste disposal problems. As well as the considerable savings associated with immobilised enzymes (i.e. reuse of the enzyme), it is also thought that immobilised enzymes are more effective in treating highly-contaminated phenolic waters. Davies and Burns (1990) compared the treatment of phenolic wastes by three immobilised enzymes with their soluble counterparts. Entrapment of HRP in alginate beads improved the efficiency of colour removal from pulp mill effluent by 132-fold, however, the system was not suitable for continuous use as the enzyme was rapidly released into solution. Glass beads have also been used with HRP incorporated onto them by precipitation of enzyme solution with glass powder. This methodology was used for the determination of phenolics and aromatic amines in organic solvents containing hydrogen peroxide. This technique could have potential applications as a packing in a reactor for the removal of phenols from aqueous effluents (Kazadijian et al.,1986). HRP immobilised onto a CNBr-Sephadex 4B column with 1 mM HCl has been shown to improve the removal of colour from kraft effluent. Removal is enhanced by a factor of 2.6; thus it would appear that the removal of soluble phenolic compounds is increased by the immobilisation of the enzyme making it more cost effective (Ferrer et al., 1991).

Recently a system was developed for use with the HRP/H_2O_2 system of depollution. This involved adding talc at the start of the reaction. Although the HRP was not actually immobilised onto the talc, it did appear to prevent inhibition of the enzyme by the oxidation products. The reaction products are absorbed onto the talc which prolongs enzyme, degradative action. (Arsequel and Baboulene, 1994). Siddique et al. (1993) attached HRP onto three different reactor matrices; cellulose filter paper, nylon balls and nylon tubing. Immobilisation onto cellulose filter paper was accomplished by

coupling the periodate-oxidised enzyme to previously oxidised filter paper. It was noted that over 80% removal efficiency was obtained for 4-chlorophenol as long as the enzyme activity was not limiting in the reactor.

1.3.6 Phenol removal from Aqueous Solution using Soluble Peroxidase

Enzymes have been used as biocatalysts for many years. In fact the industrial use of enzymes in processes such as the manufacture of cheese products , bread, beer and wine dates back to the early 1900's. Enzymes can distinguish between closely related compounds (hydroxytoluenes in the case of HRP) (Siddique et al., 1993), and have also been used for the detection and quantitation of various compounds (HRP can detect phenol concentrations of 10^{-7} M) Gazaryan et al.(1994). They can catalyse reactions over a wide temperature (0-110 °C)and pH range 2-14.

Various enzymes have been shown to perform similar reactions, such as the removal of amines and phenols from aqueous solution. Phenols can be removed from contaminated waters by the biocatalytic oxidative process of HRP. Fungal chloroperoxidase and soybean peroxidase have also been demonstrated as highly effective in removing phenolic contaminants (Carmichael et al., 1985 and Pokora, 1993). While the fungal chloroperoxidase was as effective as HRP in removing phenol and cresol, HRP appeared to be more capable of oxidising 2,3 and 2,6-dimethylphenol. The fungal chloroperoxidase displayed a greater affinity for 3-chlorophenol (Carmichael et al., 1985). A fungal chloroperoxidase isolated from *Coprinus macrorhizus* was studied for the ability to catalyse the same reactions as HRP and removed in excess of 98% phenol when mixed with peroxidase. Spiker et al. (1992) reported on a *Streptomyces viridosporus* variant of a lignin peroxidase which could oxidise phenols but was unable to remove non-phenolics. 4-aminoantipyrine was included in the reaction mixture to act as marker for lignin peroxidase activity. In the future, it may be possible to use recombinant HRP for the degradation of phenolic waste. Smith et al., (1992) have produced a recombinant HRP that is catalytically similar to plant HRP. The recombinant peroxidases are, however, expensive to produce but could include mutations that would be beneficial to a particular environmental application.

1.3.7 HRP Mechanism of Action on Phenolics

The one electron oxidation of aromatic substrate (AH_2) catalysed by peroxidase can be summarised by the following mechanism:

$$E + H_2O_2 \rightarrow E_i + H_2O \quad (1)$$

$$E_i + AH_2 \rightarrow E_{ii} + {}^{\bullet}AH \quad (2)$$

$$E_{ii} + AH_2 \rightarrow E + {}^{\bullet}AH + H_2O \quad (3)$$

The native enzyme (E) is oxidised by a reaction with peroxide to form an active intermediate called Compound I (E_i). Aromatic compounds are oxidised in the active site of Compound I. The resulting free radical, is released from the catalytic site leaving the enzyme in the Compound II state (E_{ii}). Compound II is available to oxidise a second aromatic molecule, releasing a second free radical into solution and returning the enzyme to its native resting state. The free radicals generated react to form polyaromatic products which, due to their decreased solubility can be easily precipitated out of solution. Soluble polymers can return to the active site for reaction resulting in formation of larger polymers with further reduced water solubility (Nicell et al., 1993). The rate of phenol oxidation by Compound II is known to be higher than that of Compound I (Sakurada et al., 1990). One molecule of HRP destroys approximately 10^3 molecules of phenol in its life time (Klibanov et al., 1983). Two free radicals are formed for every H_2O_2 molecule consumed. Therefore, assuming that the resulting dimer is completely soluble in water, the stoichiometric ratio of peroxide consumed per aromatic molecule precipitated would be 1:2. However, the precipitation of most phenols follows a 1:1 stoichiometry between peroxide and phenolic substrates, due to repeated reactions of soluble polymers to form trimers, tetramers or larger molecules which precipitate readily (Nicell, 1994). The concentration of peroxide required for the complete removal of aromatics is an important consideration. Removal efficiencies increase with H_2O_2 concentration only up to a certain point (Aitkin et al., 1994). It has been demonstrated that there is a loss of peroxidase activity when HRP is stoichiometrically deficient to H_2O_2 (Arano et al., 1992). Thus, it is better to use large amounts of peroxide at the beginning of the reaction when H_2O_2 is consumed rapidly and lesser amounts in the later stages (Pokora & Johnson, 1992).

1.3.8 Biodegradation of Aromatic Compounds

It appears that the structure of a pollutant affects its removal efficiency. Electron-donating substituents at the *m*-position have a greater removal efficiency than those in *o*- or *p*-positions. An increase in hydrophobicity also markedly improves the removal efficiency. Some phenolic compounds and aromatic amines have higher removal efficiencies than others. Phenols such as *o*-, *m*- and *p*-nitrophenol, *p*-cyanophenol and pyrogallol fail to precipitate following treatment with the HRP/H_2O_2 system. There are two factors proposed to explain this. First, the reactivity of these compounds towards hydrogen peroxide and peroxidase, and second, compounds such as napthols, for example, are removed rapidly as their polymeric products are more hydrophobic (i.e. less water soluble) resulting in greater precipitation from solution (Klibanov et al., 1980). Phenols with high removal efficiencies have been shown to aid in the precipitation of the more difficult compounds. Phenols have been shown to be more readily oxidised in the presence of compounds such as 3,3'-dimethoxybenzidine, *o*-dianisidine and 8-hydroxyquinoline (Klibanov et al., 1980 and Klibanov, 1982). Free radicals formed from benzidine molecules attack and precipitate phenols. This mechanism has been reported to work with non-phenolic and non-amine compounds. Also, ortho- and meta- substituted cresols are removed at a greater rate than p-cresol at pH 4.0 (Siddique et al., 1993 and Klibanov, 1982). Carmichael et al. (1985) noted that the removal efficiencies of phenol and cresol were similar but monochlorophenols were most readily oxidised by HRP and a fungal chloroperoxidase. A manganese peroxidase which is capable of oxidising pentachlorophenol more efficiently than phenol has been described.

CHAPTER 2

MATERIALS AND METHODS

Aldrich, Chemical Company, Inc., Milwaukee, USA.

Acetic acid

Acid red 151

Aluminium potassium sulfate dodecahydrate

1,2,4,- Benzenetricarboxylic Anhydride (trimellitic anhydride)

1,2,4,5,-Benzenetetracarboxylic dianhydride (Pyromellitic anhydride)

Chicago sky blue 6B

Citric acid crystals

Dipotassium hydrogen orthophosphate

Disodium hydrogen phosphate dihydrate

Direct blue 71

Hydrochloric acid

Hydrogen peroxide

4 - Hydroxycinnamic acid

7 - Hydroxycoumarin

4 - Hydroxybenzsulfonic acid

Magnesium chloride, 6-hydrate

Methyl orange

Potassium chloride

Potassium dihydrogen orthophosphate

Potassium ferrocyanide

Sodium-dihydrogen orthophosphate dihydrate

Trifluoroacetic acid (TFA)

Tween 20

Vanillin

Adipic acid dihydrazide agarose (AADH-A)

Alginic acid

Sigma Chemical Company, Poole, Dorset, England

Ammonium persulfate

Adipic acid dihydrazid-agarose (AADH-A)

2, 2'- Azino-bis (3- Ethylbenzothioline - 6 - sulfonic acid) (ABTS)

Borax

Bovine pancreatic trypsin (EC 3.4.21.4) type III

Bromophenol blue

Calcium chloride

Catalase (E.C.1.11.6, 7,080 units mg^{-1} protein)

Coomassie Brilliant Blue R-250

Cyanogen bromide

Diacetyl (2,3 - butanedione)

DL - dithiothreitol (DL - DTT)

Endoproteinase - Lys - C

Ethylene glycol - bis(succinic acid N-hydroxysuccinimide ester) (EG-NHS)

Glycine

Glyoxal

Guanidine hydrochloride

Lysozyme

Molecular weight markers (ml.wt. 14,200-66,000)

3-[N-Morpholino] propanesulfonic acid (MOPS)

2-(N-Morpholino) ethanesulfonic acid (MES)

Nile Red

C - peptide (insulin chain C)

Peroxidase (E.C. 1.11.1.7)

Phthalic anhydride (1,3-iso benzofurandione)

Pyrogallol

Sephadex ™ G-25

Sodium hydroxide

Sodium bicarbonate

Sodium chloride

Sodium dodecylsulfate (SDS)

Soybean trypsin inhibitor

N,N,N',N'-tetramethyl ethylenediamine (TEMED)

3,3'5,5-tetramethylbenzidine (TMB),

2,4,6-trinitrobenzenesulphonic acid (TNBS)

Tris-(hydroxymethyl) aminomethane (Tris)

L- Tryptophan

Urea

Labscan Ltd., Dublin, Ireland,

Acetone

Dimethylformamide (DMF)

Dimethylsulphoxide (DMSO)

1,4-Dioxan (DXN)

Methanol

Tetrahydrofuran (THF)

Romic Ltd.

Methyl ethyl ketone (Butanone)

Cal Biochem.

Tris(2-carboxyethyl) phosphine (TCEP), Hydrochloride

Whatman Ltd., Maidstone, England.

pH indicator paper

Costar

96 well flatbottom polystyrene microtitre plates

Pierce Chemical Co., Illinois, USA.

Bicinchoninic acid (BCA),protein assay kit

Biozyme

Peroxidase (E.C. 1.11.1.7)

Boehringer Mannheim, Germany,

Peroxidase (E.C. 1.11.1.7)

Riedel-de-Haen AG., Germany

2-Mercaptoethanol

Dr. Andrew Smith, Sussex University, Brighton, UK.

Recombinant HRP C

Pharmacia

Sephadex G-25

Prepacked Sephadex PD10 columns

1ml cation exchange column (mono S HR5/5)

2.2 Quantitative Enzyme Activity Assay

2.2.1 TMB Assay

3,3,'5, 5,'-Tetramethylbenzidine dihydrochloride (TMB) was used as a reducing substrate in routine activity determinations. This colorimetric assay was developed by Bos et al (1981) and Gerber et al (1985) and is similar to the method used by Ryan et al (1994). HRP was prepared at a concentration of $1mg.ml^{-1}$ in 100mM phosphate buffer, pH 7.0. Serial dilutions from this stock were made up in 10 mM phosphate buffer pH 7.0 + 0.002% (v/v) Tween 20 to a final assay concentration of $10\mu g\ ml^{-1}$. TMB was used at a concentration of $0.1g\ L^{-1}$ in powder form.

Dimethylsulphoxide (DMSO) (at 2% final volume) was used initially to dissolve TMB powder which was then added to 10ml buffer. The buffer employed was 100mM citrate, pH 5.5. Immediately prior to assay, 30% v/v H_2O_2 was added to the TMB solution to give a final concentration of 0.03%.

Four 50µl aliquots of 10 $\mu g\ L^{-1}$ HRP were dispensed into a 96 well microtitre plate. This was then equilibrated at 25° C in a Titertek Multiscan plus plate reader (ICN Laboratories). 150µl of buffered substrate solution was dispensed into each well to start the reaction. Blank wells contained 50µl of 100mM phosphate buffer, pH 7.0. The reaction was allowed to proceed for 2 min and the A_{620} values were determined (Liem et al., 1979). A blue colour developed in the wells as the reaction proceeded. Each activity determination was the mean of 4 wells.

2.2.2 ABTS Assay

The method was based on that of Smith et al (1990). ABTS was used as substrate in steady-state kinetic determinations. Enzyme samples $100\mu g\ L^{-1}$ (20µl) were added to a cuvette (1cm, pathlength) containing 0.91 mM ABTS in phosphate citrate buffer (51 mM Na_2HPO_4, 24mM citric acid, pH 5.0) and 2.5 mM H_2O_2 in a final volume of 1.0 ml. The rate of change of A_{405} was measured immediately in a Unicam UV/visible spectrophotometer UV2 at 25 °C.

2.3 Protein Determination

2.3.1 Bicinchoninic Acid (BCA) Assay

2.3.1.1 Standard Protocol

This method used the Pierce BCA protein assay and was based on the method of Smith et al (1985). A set of standards was prepared by diluting the stock BSA (2mg ml^{-1}) provided with the kit in the range 200µg ml^{-1} to 1,800 µg ml^{-1}. A 10µl volume of each standard, sample, blank and unknown were pipetted into the appropriate microtitre wells (four replicates). Working reagent (200µl) was added to each well and mixed. The plate was then covered and incubated at 37 °C for 30 min. Absorbance at 560nm was read on a Titertek Multiscan plus MK II. The concentration of the unknown protein sample could then be obtained from the standard curve.

2.3.1.2 Enhanced Protocol

This method was performed as per section 2.3.1.1., with the following modifications. A) The calibration standards were used in the range 50µg ml^{-1} to 250µg ml^{-1} BSA and B) the plate was incubated at 60 °C for 30 min prior the reading the absorbance at 560 nm.

2.3.2 Extinction Coefficients.

The concentration of HRP could also be determined by dilution of a sample and measurement of its Soret millimolar extinction coefficient at 402 nm =102mM^{-1} cm^{-1}. (Smith et al., 1992).

2.3.3 Determination of HRP Activity using Pyrogallol

The method based on that of Pokora & Johnson, (1992) was used to estimate the HRP activity. 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.

2.4 Chemical Modifications

2.4.1 Modification with Diacetyl and Glyoxal

Arginine can be modified by dimeric or trimeric 2,3-butanedione (diacetyl) and glyoxal. The modification protocol was based on the methods of Gripon and Hofmann (1981) and Yankeelov (1972). To a 1mg ml^{-1} solution of HRP was added a 15 - or 10-fold molar excess [a "molar excess" indicates an excess of reagent over appropriate HRP R-groups] of diacetyl or glyoxal respectively with stirring. The sample was then incubated at 25 °C for 6 hours. In the case of diacetyl, this reaction proceeded in the dark. The reaction was terminated by overnight dialysis in phosphate buffer (10mM, pH 7.0) at 4°C. Prior to this, the procedure had been optimised with respect to a number of variables including

1. exposure time of reagent to HRP.
2. concentration of reagent used in the reaction
3. presence or absence of light.

Reaction temperature (25°C) and the pH of solution (pH 7.0) were kept constant.

2.4.2 Nitration of Tyrosine Residues with Tetranitromethane (TNM)

The method resembled that of Mozhaev et al (1988). 1ml of TNM (10-200 fold molar excess) was added to 1ml of a 1 mg ml $^{-1}$ HRP solution and incubated for 2h at room temperature with stirring. Excess reagent was removed by G-25 gel filtration (Section 2.7).

2.4.3 Crosslinking with N-hydroxysuccinimide Esters

The bifunctional reagent ethylene glycol N-hydroxysuccinimide ester (EG NHS) was used. The method used was based on that of Ryan et al. (1994). To 1 ml of 1mg ml^{-1} HRP, in 10 mM phosphate buffer, pH 7.0, was added 1mg of N-hydroxysuccinimide ester dissolved in 5% (v/v) DMSO. Modification proceeded at room temperature for 20 min and was terminated by the addition of an equal volume of cold 100mM Tris HCl pH 7.0. Alternatively, the samples were centrifuged through a sephadex G-25 column (Section 2.7) to remove any excess reagent. Samples were then diluted to standard assay concentrations in 10 mM phosphate buffer, pH 7.0 + 0.002% (v/v)Tween 20 and assayed for initial recoveries and % relative catalytic activity

2.4.4 Covalent Modification with Cyclic Anhydrides

The protocol used was based on that of Mozhaev et al (1988). To 900µl of 0.15mM horseradish peroxidase was added, with stirring, 100µl of 15mM cyclic anhydride [Phthalic, Trimellitic or Pyromellitic Anhydride] in DMSO i.e. 100 molar excess. The reaction proceeded at 4 °C for 2.5 h, and was terminated by G-25 gel filtration (Section 2.7) to remove excess reagent.

2.5 Determination of Amino Groups

2.5.1 Trinitrobenzensulphonate (TNBS)

Amino group determination was performed using trinitrobenzesulphonate (TNBS) according to the method of Fields (1971). 250µl of 5µM HRP was added to 500µl borate buffer (100mM $Na_2B_4O_7$ in 100mM NaOH, pH 9.2). 87µl of 1M TNBS was then added. The sample, was mixed and allowed to equilibrate at room temperature (25° C) for 30 min. The reaction was terminated by addition of 250µl SDS (10% w/v) and 125µl 1N HCl . Absorbance at 420nm as determined against a blank (i.e. no protein) was measured. Readings were taken using a Unicam UV2 spectrophotometer. A standard curve was prepared using standards of α-N-acetyl-L-Lysine (i.e., one free amino function) in the concentration range of 0.05-10mM. All readings were carried out in triplicate and their means determined.

2.5.2 Fluorimetric Assay using Fluorescamine

This method was based on that of Bohlen et al. (1973). 50mM Sodium phosphate buffer, pH 8.0, was added to 150µl HRP. While the sample was being vigorously shaken on a vortex mixer, 500µl of fluorescamine in dioxane (0.03% w/v) was rapidly added via a syringe. Fluorescence was measured on a Perkin Elmer LS 50 fluorimeter, with the excitation wavelength set at 390nm and the emission wavelength at 475nm. Since the fluorescence obtained with some proteins changes slowly over time, measurements were made at 2 min after each reaction.

2.6 TCA Precipitation

20g of Trichloroacetic acid (TCA) was dissolved in 100ml of distilled water to give a 20% solution. 500µL of this was added to 500µl sample in an ampoule to give a final concentration of 10% TCA. The ampoules were left on ice for 30 min and then centrifuged at 3,000 rpm until a precipitate appeared. The TCA was carefully removed and the pellet washed with acetone, resuspended and centrifuged again. The washing cycle

was repeated 3 times, and finally the pellet was resuspended in 30-50μl of solubilisation buffer (Section 2.21).

2.7 G-25 Gel Filtration

The method was similar to that of Helmerhorst and Stokes (1980). Sephadex G-25 (10g) was swollen in 100ml 10mM phosphate, pH 7.0, (or other buffer depending on the application). The Sephadex slurry was stirred continuously for a minimum of 3 h, then 20ml was poured continuously (by means of an adapter) into a 10ml column. The column was then capped and stored until required. Two methods of desalting were used: the first involved centrifuging the column for 30 sec at 2,000 rpm to produce a semi-dry column to which the sample was applied. The column plus sample was then centrifuged as above and the eluant collected. The second method was the traditional method where the sample was applied to the buffered column, followed by eluting buffer and fractions were collected.

2.8 Thermoinactivation Studies

2.8.1 Temperature Profile

HRP samples (1ml of 10μg L^{-1}) in 10mM phosphate buffer, pH 7.0, were incubated for 10 min over a range of temperatures (30 - 80°C) for 10 min. The samples were cooled rapidly on ice and assayed using TMB as in Section 2.2.1 for relative catalytic activity, (i.e relative to a control held at 4° C). A temperature profile of the remaining activity against temperature was then plotted.

2.8.2 Thermoinactivation Studies

HRP samples (1ml of 10μg L^{-1}) in 10mM phosphate buffer, pH 7.0, were incubated at 65°C for 1 hour. Aliquots were withdrawn at 2, 5 and 10 min and then at 10 min intervals and stored on ice. Each sample was then assayed under the standard TMB assay conditions (Section 2.2.1) and percentage relative catalytic activity (RCA)

determined at each point, (i.e., 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 the HRP activity to decline to 50% of its initial value) were estimated by inspection of the plots of % RCA versus time.

2.8.3 Comparison of MOPS Buffer vs Phosphate Buffer

1ml samples HRP 10μg L^{-1} in 10mM MOPS buffer and in 10mM phosphate buffer, pH 7.0 were incubated at 65 °C for 1h. Aliquots were withdrawn at 2, 5, and 10 min initially and then at intervals of 10 min onto ice. After the 60 min sample had been taken, all the samples were assayed simultaneously by the TMB assay.

Samples exposed to 65°C as above were also assayed for residual activity immediately following removal using the ABTS assay (Section 2.2.2).

2.8.4 Effect of $CaCl_2$ and EDTA on HRP in Phosphate or MOPS Buffer

Samples of HRP in 10mM phosphate buffer, and 10mM MOPS buffer, pH 7.0 were incubated in 1mM $CaCl_2$ or 1mM EDTA and a thermoinactivation study was carried out as described in Section 2.8.2. The residual activity for each set of samples was determined and a thermoinactivation curve plotted. The half-life for each set of samples was obtained and compared.

2.9 Enzyme Stability in Organic Solvents

Organic solvent profiles of HRP samples were carried out at 25 °C. Samples of HRP (10μg L^{-1} final concentration) were incubated in 10mM phosphate buffer, pH 7.0, and organic solvent in the range 0-90% (v/v) for 60min. Aqueous control samples were included throughout. Four 50μl aliquots were withdrawn onto ice after 60 min and assayed for residual catalytic activity as described in Section 2.2.1.

2.10 Thermostability of Enzymes in Organic Solvents

To demonstrate the combined effects of organic solvents and elevated temperatures, the procedure in Section 2.9 was followed. However, the samples were incubated at 65 °C for 10 min. The % activity remaining was then compared to samples containing no solvent (aqueous control) and incubated at 25 °C for 10 min.

2.11 pH Profiles

100mM buffer solutions, at pH 6.0, 7.0, 8.0, 9.0 and 9.5 were prepared with acetic acid-sodium acetate buffer, phosphate buffer, Tris-HCl buffer and glycine-NaOH buffer respectively. Native and modified HRP were diluted to assay concentration (10μg L^{-1}) in each of the buffers and incubated for 30 min at room temperature. Catalytic activity was then assayed as described in Section 2.2.1.

2.12 Preparation of Apo-Peroxidase

The haem component of the HRP was removed using a modification of Yonentani's (1967) method. HRP (5-10 mg ml^{-1}) was dissolved in 1ml of 100mM ice cold phosphate buffer, pH 7.0 and stored on ice for 1 min. The pH was adjusted to 1.8 by dropwise addition of 6 M HCl. Immediately, an equal volume of ice cold butanone was added. The mixture was shaken vigorously for 30 sec and placed on ice for a further 1 min. The deeply coloured upper layer was siphoned off from the colourless lower layer using a glass Pasteur pipette. The remaining aqueous phase was treated twice more with butanone to obtain a completely clear solution. The sample was then passed through a Sephadex G-25 column (Section 2.7) to remove any excess butanone and to re-equilibrate the sample to pH 7.0 in 10mM phosphate buffer.

2.13 Endoproteinase Lys-C (Endo Lys-C) Digestion

This method was based on those of Steffens et al. (1982) and Amersham international (personal correspondence). 100μl (500μg ml^{-1}) of apo/holo HRP in 100mM ammonium bicarbonate buffer, pH 8.5, + 0.1% (w/v) SDS was incubated with 4μl of endo

Lys-C at 37 °C for 4 h. A further 2µl of endo Lys C was added and incubated for a further 16 h. Reaction was terminated by mixing in a 1:1 ratio with solubilization buffer (Section 2.21). Samples were then electrophoresed (Section 2.21) on a 12% polyacrylamide gel, stained with Coomassie Blue and the positions of bands noted. This procedure was also carried out with HRP prepared in 100mM phosphate buffer, pH 7.0.

2.14 Cyanogen Bromide Cleavage

Cyanogen bromide cleavage was performed essentially as described by Gross and Witkop (1967). A 100-fold molar excess of cyanogen bromide over HRP methionines in 0.1N HCl was added to the apoenzyme/holoenzyme and the reaction allowed to proceed in the dark for 24 hours. (Note: all cyanogen bromide procedures were carried out in the fume hood). At the end of the reaction period, the pH of the samples were adjusted to neutral by the dropwise addition of NaOH. Samples were then diluted 1:1 with solubilization buffer and boiled for 2 min in preparation for loading onto the SDS gels. (Section 2.21).

2.15 Tryptic Digestion of HRP

The apo/holoenzymes were exposed to trypsin for 2 h at 37 °C at an enzyme substrate ratio of 1:100 (standard conditions). This method was based on the method of Welinder (1979). After the incubation period, the reaction was terminated by addition of an excess of soyabean trypsin inhibitor, then the samples were diluted 1:1 with solubilization buffer (Section 2.21).

2.16 Unfolding of HRP

2.16.1 Stability Towards Urea

A stock solution of 8M Urea was prepared according to the method of Pace et al. (1990). This was then used to incubate HRP samples in the range 0 - 8M Urea. Samples were incubated for periods of up to 2 h at room temperature and were then assayed for residual activity as described Section 2.2.1. Samples of HRP were also

incubated in 0 - 8M Urea in the presence of 1mM EDTA for up to 2 h at room temperature before being assayed for residual activity.

2.16.2 Stability Towards Guanidine Hydrochloride (GdCl)

The degree of unfolding of native EG, and PA HRP upon exposure to guanidine hydrochloride (GnCl) was determined according to the method of Pace et al. (1990). A stock solution of 10M GdCl was prepared by weighing 9.557g GdCl into a beaker and adding 1.5ml of 1M HCl and 3.5ml of 100mM phosphate buffer, pH 7.0. This was allowed to dissolve and the pH was then checked and adjusted to 7.0. A stock solution of 100μg L^{-1} of HRP was prepared. The percentage of residual activity was then determined in the range 0-10M GdCl (2M increments) and in the range 0 - 5M GdCl (0.5M increments). The HRP end concentration in all cases was 10μg L^{-1} (standard assay concentration). After preparation, all solutions for measurement were incubated at room temperature for 60 min and their remaining catalytic activities were assayed (Section 2.2.1).

2.16.3 Stability Towards Reducing Agents

Stock solutions of EDTA and TCEP of 1000μM and 100μM respectively were prepared. Unfolding curves within these concentration ranges were prepared as for GdCl, the difference here being that all the solutions contained 4.5M GdCl as standard. The concentration ranges in subsequent experiments were decreased or increased according to the results obtained in the activity assay and these further samples were assayed for remaining activity. Mercaptoethanol was added to a GdnCl-treated stock of HRP to a final concentration of 150μM. This was incubated at room temperature for 60 min and its % RCA then determined.

2.17 Fluorescence Spectroscopy (unfolding at high temperatures)

2.17.1 Tryptophan Fluorescence

Tryptophan fluorescence intensity of native HRP and derivatives was monitored from 300-400 nm (emission wavelength). The excitation wavelength was set to 283 nm, with slit widths of 10.0nm for both excitation and emission. (Ryu and Dordick, 1992). All experiments were carried out using a Perkin Elmer LS 50 fluorescence spectrophotometer. The peroxidase concentration was 3.5μM in all solutions studied. A stock solution of L-tryptophan (prepared in 3.5μM peroxidase or distilled water) was used to construct a standard curve of tryptophan fluorescence in the range 2-10 μM. Peroxidase samples were continuously scanned in a cuvette holder maintained at 65 °C by a circulating water bath. A temperature probe was inserted into the cuvette to determine the exact temperature at each scan. The data from the scans collected by the LS 50 software was imported into a PC based graphics package (Sigma Plot) and the fluorescence maxima and wavelength shifts calculated.

2.18 Hydrophobic Probe (Nile red)

The method of Sackett and Wolff (1987) using Nile Red as a hydrophobic probe was followed with some slight adjustments. 100 mM MES buffer, pH 6.9, containing 1 mM EGTA and 1 mM $MgCl_2$ was prepared. A stock solution of 250 mM Nile Red in DMSO was prepared. HRP solution (approximately 2.0 mg ml^{-1}) in 10 mM phosphate buffer, pH 7.0, was placed in a water bath and held at a series of temperatures ranging from room temperature to 85 °C. At regular intervals, 0.4 ml aliquots were removed, added to 0.8ml MES buffer and plunged in ice for 1 min. Eight microlitres of stock Nile Red (2μM) was added and mixed. Samples were allowed to stand for 15 min before being read. Fluorescence measurements were performed on a Perkin Elmer LS50 fluorescence spectrophotometer. The sample was excited at 550 nm and the emission scan was monitored from 560 nm to 700 nm. Excitation and emission slit widths were set at 5 nm.

2.19 Hydrogen Peroxide Stability

Hydrogen peroxide stability was determined according to the method of Welinder (1993). HRP undergoes a well characterised 2-electron oxidation on addition of hydrogen peroxide to give a high oxidation-state intermediate called Compound I. Hydrogen peroxide stability was measured as the irreversible inactivation of HRP Compound III (scheme 1.2). The absorbance at 405 nm was measured over time, and the time required for this to decrease to half the original value was defined as the half life. The test solution was composed of 10 mM phosphate buffer, pH 7.0, 5.8μM HRP and 4.4 mM (or 800 times molar excess) hydrogen peroxide, measured in a temperature controlled spectrophotometer attached to a 25 °C water bath.

2.20 Immobilisation of HRP and recombinant HRP C

Before immobilisation the gel was washed to remove the preservatives and stabilisers. 20 ml of commercial AADH-A was washed with 4 times with 20 ml water and 6 times with 25 ml buffer. After this treatment 0.2 ml of AADH-A was added to 1ml HRP (1mg ml^{-1}) (Moreno & O'Fagain, 1996). This mixture was stirred continuously at room temperature for 4 h. The sample was then centrifuged at 4,500 rpm for 10 min. The supernatant was removed and the pellet washed with 1 ml 100mM phosphate buffer, pH 7.0, followed by 10mM MOPS, pH 7.0. This procedure was repeated twice, and the supernatants were pooled after each cycle. Pooled supernatant samples and immobilised fractions were assayed for residual activity.

2.20.1 Immobilisation in Calcium Alginate

The immobilisation of HRP samples in alginate beads was carried out according to the procedure outlined in "methods in enzymology" vol. 135. A 1.5% (w\v) solution of alginic acid was prepared in distilled water. The alginic acid gel was allowed to form over a period of two hours at room temperature with continuos stirring. 500μL (100μg L^{-1}) of native or modified HRP was added to 5 ml of alginic acid gel and stirred

for 10 min. The suspension of enzyme/sodium alginate was passed dropwise into a solution of 100mM $CaCl_2$, from a height of 10cm, using a syringe with an outlet diameter of 1mm. Bead formation was instantaneous and beads had to be removed from the site of bead formation to avoid clogging. Beads were left in the $CaCl_2$ for 20 min to allow gelation of the beads to occur. The beads were then semi-solid and easy to handle and could be stored in an air tight container until required for use.

2.21 SDS-Polyacrylamide Gel Electrophoresis. (SDS-PAGE)

SDS-PAGE was performed to examine the band fragments produced by endo Lys-C digestion, cyanogen bromide cleavage and tryptic digestion. The method followed was that of Laemmli (1970).

2.21.1 Solution Preparation

Table 2.1 gives details on the preparation of gels using the following solutions.

Solution A

29.2g Acrylamide and 0.8g N-N'methylene-Bis.Acrylamide in 100ml distilled water.

Solution B

22,6g Tris in 100ml distilled water and pH adjusted to 8.8, (1.87M)

Solution C

6.06g Tris in100ml distilled water and pH adjusted to 6.8,(0.5M).

Solution D

0.1g Ammonium Persulphate in 1ml distilled water, 10% A.P (w/v.).

SDS

0.1g SDS in 1ml distilled water, 10% SDS (w/v).

Running Buffer

3.028g Tris - (0.025M), 14.4g Glycine - (0.192M), 1g SDS.- (0.1% w/v)

in 1 L distilled water and the pH adjusted to 8.3.

Solubilisation Buffer

0.969g Tris , 10ml Glycerol , 10ml Bromophenol blue , 5ml Mercaptoethanol , 2% SDS

Staining Solution

2.5g Coomassie Brilliant blue , 500ml Methanol , 100ml Acetic acid in 1 L filtered distilled water.

Destaining Solution

250ml Methanol , 70ml Acetic acid in 1 L distilled water.

2.21.2 Sample Preparation

The samples and molecular weight markers were mixed 1:1 with solubilisation buffer and boiled for 2 min. 10-15µl of each sample was then loaded onto a prepared minigel under running buffer. A constant current of 20-25mA per gel was applied with a running time of approximately 3 h (or until the dye front was at the end of the gel.) A Consort minigel system C E443 power pack was used.

Molecular Weight Markers (Sigma);.	**Molecular Weight (Da)**
Albumin, bovine serum	66,000
Albumin, egg(ovalbumin)	45,000
Glyceraldehyde -3phosphate dehydrogenase	36,000
Carbonic anhydrase	29,000
Trypsinogen, Bovine pancreas	24,000
Trypsin inhibitor, soybean	20,100
A-Lactalbumin,Bovine milk	14,200

Table 2.1. Preparation of Gels for SDS-PAGE

TYPE OF GEL	RESOLVING (10%)	RESOLVING (12%)	RESOLVING (15%)	STACKING (3%)
STOCK SOLUTION	VOLUME (ml)	VOLUME (ml)	VOLUME (ml)	VOLUME (ml)
Water + 3.3% SDS	9.0	7.0	4.0	7.8
Solution A	10.0	12.0	15.0	1.0
Solution B	11.2	11.2	11.2	–
Solution C	–	–	–	1.25
Solution D	0.15	0.15	0.15	0.075
TEMED *	0.05	0.05	0.05	0.05

* TEMED acts as a catalyst for gel polymerisation, so it is added last.

Gels were placed in the staining solution for 15-20 min, then transferred to destaining solution which was changed at intervals until a clear background was obtained.

2.22 Calibration of the Biosep SEC -3000 Column with Molecular Mass Standards

A Biosep-3000 column was equilibrated with 50ml 100mM potassium phosphate, pH 7.4, at a flow-rate of 0.5ml min^{-1}. Standards applied to the column included cytochrome C (12,400Da), carbonic anhydrase (29,000Da), BSA (66,000Da), alcohol dehydrogenase (150,000Da) and β-amylase (200,000Da). 5μL injections of 2mg ml^{-1} of each standard were applied and eluted from the column with 15ml equilibration buffer at a flow rate of 0.5ml min^{-1}. The elution of each standard was monitored at 214nm, 280nm and by continuous scanning of the column eluant using the photo diode array (PDA). Peak detection and retention time determination for each standard was achieved using Beckman System Gold peak integration software. The elution volume (V_e) was calculated as the retention time (minutes) of each standard multiplied by the flow-rate (0.5ml min^{-1}). A plot of log molecular mass versus V_e/V_o of each standard was prepared, and a calibration curve for the column was thus constructed.

2.22.1 Estimation of Relative Molecular Mass of Digested Enzyme Fragments

5μl injection of the native enzyme and the two modified forms (EG-NHS and PA HRP) and the cyanogen bromide fragments were applied and eluted from the column with 15ml equilibration buffer at a flow-rate of 0.5 ml min^{-1} while 0.2 ml fractions were collected. The plots obtained were analysed visually for evidence of fragmentation.

2.23 Purification of Native HRP and Derivatives

The peroxidase samples were equilibrated into 20mM Na^{+}- acetate buffer pH 4.3. All samples were checked for pH after equilibration as the pH is vital for binding to the FPLC column. The samples were then loaded on to a 1ml cation exchange column (Mono S HR5/5: Pharmacia FPLC system) equilibrated with 20mM

Na^+- acetate buffer pH 4.3. Elution was with a linear NaCl gradient, 0-0.3 M over 20ml, at a flow rate of 1 ml min^{-1}. Fractions were collected every 1 ml. One major A_{280} peak with a shoulder on either side was obtained at 130 mM NaCl and contained active peroxidase. The aliquot containing the major peak was put through the FPLC system a second time and one major A_{280} peak was obtained. Samples were transferred into 25 mM MOPS pH 7.0 by gel filtration in a 9 ml Sephadex pre packed column (Pharmacia LKB Biotechnology PD-10). The concentration of each sample was then determined using the extinction coefficient (Section 2.3.2).

2.24 Benzhydroxamic Acid (BHA) Binding Studies

The method of Smith et al. (1992) was used for these studies. Difference spectra in the Soret region (350 - 450 nm) of the enzyme with and without BHA were recorded in a 1cm quartz microcuvette in 10 mM phosphate buffer, pH 7.0. 400µl of buffer was placed in both the reference and the sample cell of a Unicam UV2 dual beam spectrophotometer and a baseline recorded. Then the sample cell was replaced with 400µl of native or modified HRP (4µM). Successive 1µl aliquots of 400 mM stock BHA were added to the sample and spectra were recorded over the range 1.2 - 45 µM BHA. The dissociation constant (K_d) of the HRP-BHA complex was determined by fitting the data (using a weighted least-squares error minimisation procedure) to the equation

$$A = 2A_\infty L/\{(L + K_d + P) + [(L + K_d + P)^2 - 4PL]^{1/2}\},$$

where the absorbance change at 408 nm (resulting from benzhydroxamic acid of concentration L binding to a total protein concentration P) is determined, whilst allowing the remaining K_d and maximum absorbance change at saturation (A_∞) to float. No buffer was added to the reference cuvette, as the total volume of substrate added never exceeded 2% of the starting sample volume and effects due to sample dilution were compensated for during calculation of the binding constants.

2.25 Steady State Kinetics on HRP and Derivatives

2.25.1 Kinetics using ABTS

The steady state kinetics of native and modified forms of HRP (EG-NHS and PA HRP), were determined using ABTS as substrate based on the method of Childs and Bardsley (1975). A 10 mM stock solution of ABTS was prepared in 200mM dihydrogen phosphate, 100mM citric acid buffer, pH 5.0. A 100mM H_2O_2 stock was prepared in ultrapure water (Concentration was checked spectrophotometrically; $\varepsilon_{H2O2} = 43.6\ M^{-1}cm^{-1}$). An enzyme stock solution 100μg L^{-1} was prepared in 10mM phosphate buffer pH 7.0. 100μl of substrate and 10μl of H_2O_2 was added to the cuvette and the volume adjusted to 990μl with buffer (i.e. buffer used to dissolve ABTS). This was allowed to equilibrate at 25 °C for 2 min. 10μL of enzyme was added and the reaction was monitored at 405nm using a Unicam UV2 spectrophotometer for 3 min. The temperature was maintained at 25 °C during the assay by a Julabo U3 circulating water bath connected to the spectrophotometer. Reference cells containing reaction mixture with no enzyme were used as blanks for each concentration of substrate. $\Delta A\ min^{-1}$ values were recorded for each sample. All samples were assayed in triplicate and their average calculated. The K_m and k_{cat} values for both the native and the modified HRP were determined using the Enzfitter programme (Biosoft, Cambridge, U.K.) and also using the Direct Linear Plot within Sigma Plot.

2.25.2 Kinetics using Ferrocyanide

Steady state kinetics using potassium ferrocyanide as substrate were determined according to the method of Hasinhoff and Dunford (1970). Stock solutions of 10mm H_2O_2 in ultrapure distilled water, 0.9μM HRP in 10mm phosphate buffer, pH 7.0 and 10 mM ferrocyanide in 10 mM phosphate buffer, pH 7.0 were prepared. To a cuvette was added 20 -140μl of substrate, 100μl of H_2O_2 and the volume made up to 990μl with 10mM phosphate buffer, pH 7.0. This mixture was allowed to equilibrate for 1 min at 25 °C. Reaction was initiated by the addition of

10μL of enzyme to the cuvette. Reaction was monitored with a Unicam UV2 spectrophotometer by following the increase in absorbance at 420nm (due to the production of ferricyanide) for 3 min. The temperature was maintained at 25°C during the assay by a Julabo U3 circulating water bath connected to the spectrophotometer. Reference cells containing reaction mixture with no enzyme were used as blanks for each concentration of substrate. $\Delta A\ min^{-1}$ values were recorded for each sample. All samples were assayed in triplicate and their average calculated. The k_{3app} ($M^{-1}\ s^{-1)}$ from steady state kinetics was determined from the following plot,

$[HRP]_o / \upsilon$ versus $1/[Fe(CN_6)^{4-}]$

The slope of which is 1/2 k_{3app} with units of $mol^{-1}.sec^{-1}$ (Hasinhoff and Dunford, 1970).

2.25.3 Pre-Steady-State Kinetics

Pre-steady-state for HRP were monitored on a stopped flow spectrometer (model SF-55, Hi-Tech Scientific, Salisbury, UK) at 25.0 ± 0.2°C with a slit width of 0.5nm, in 10mM sodium phosphate buffer, pH 7.0. Data were recorded through an RS232 interface on a microcomputer. Rate constants were obtained by fitting recorded data to exponential functions by using a least squares minimisation procedure.

2.25.3.1 Compound II Formation Test Reaction

3.0mM HRP 100μl was mixed with 100μl of 2.7mM hydrogen peroxide (all solutions at 4°C); this was then immediately mixed with 200μl 1.65mM ferrocyanide and the absorbance spectrum scanned every 5 minutes in rapid mode from 500-300nm. From this, the purity and stability of Compound II was estimated by visual inspection of the scans obtained. When these proved satisfactory (i.e. when the isosbestic point at 424nm was apparent), then the stopped flow kinetic analyses were carried out.

2.25.3.2 Estimation of k_3

The method used was based on that of Smith et al. (1992). For each enzyme form, values of k_{obs} were measured with the following compounds: ferulic acid 4-20mM, ferrocyanide 4-20mM, Indole 3-propanoic acid 200-1000mM. Experimental traces of ΔA_{424} (the isosbestic point between resting enzyme and Compound I) against time during the reduction of Compound II by the various substrates were fitted to exponential functions. Direct mixing was accomplished within the stopped flow apparatus. All determinations were carried out within 5 min of Compound II preparation.

2.26 Recombinant HRP

The concentration of recombinant HRPC (HRP C*) was determined by measurement of the Soret molar extinction coefficient at 402 nm = 102 mM^{-1} cm^{-1} (Smith et al., 1992). HRP C* was then diluted to the optimum assay concentration (10μg L^{-1}) in 10 mM phosphate buffer/0.002% Tween 20, pH 7.0. The activity was assayed as described in Section 2.2.1. Modification of HRP C* was carried out with the bifunctional reagent ethylene glycol bis-succinimidyl succinate (EG-NHS) (Section 2.4.3).

2.27 Bleaching Effect of Native and Modified HRP on Dyes

2.27.1 Bleaching Effect at Room Temperature

The method was that of Welinder (1993). The enzyme acts by reducing the coloured substance (donor substrate) dissolved or dispersed in the reaction media, thereby generating a colourless product. This generalised reaction scheme is shown below:

$$\text{Donor substrate} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{oxidised donor} + 2\ H_2O$$

The enzyme concentration was determined as described in Section 2.3.2. The test solution was composed of 10 mM phosphate buffer, pH 7.0, 200μM hydrogen peroxide, 50 μM oxidisable substrate (accelerator) and 1 - 45 nM peroxidase. The optimal HRP

concentration for assay of each dye was determined by incubating test solutions containing HRP for 10 min in the range 1-45 nM; the bleaching effect, if any, was determined by measuring the decrease in absorbance at the wavelength appropriate for the dye and by visual inspection. The dyes used in the assay were Acid Red 151, Chicago Sky Blue 6B, Direct Blue 71 and Methyl Orange. Dye concentrations were between 4-8μM. The test temperatures were 25 °C and 65 °C. The oxidisable substrates were 4-hydroxycinnamic acid, 7-Hydroxycoumarin, 4-Hydroxybenzsulfonic acid and Vanillin.

2.27.2 Effects of Elevated Temperature on Bleaching

The bleaching effect at each temperature was monitored up to 20 min for all of the dyes with each of the accelerators. The degree of bleaching was taken as the decrease in absorbance at 490 nm for Acid Red 151, 618 nm for Chicago Sky Blue 6B, 580 nm for Direct Blue 71 and 465 nm for Methyl Orange. The λ max of each dye had earlier been determined from spectral scans in the range 350-750nm

2.27.3 Effect of Solvents on Bleaching

Vanillin was used as the accelerator for each dye. Reaction mixtures were incubated with 40% (v/v) dioxane or DMF for 10 min, and the change in absorbance determined as in Section 2.27.1 above.

2.27.4 Effects of Immobilised HRP on Bleaching

The method used was similar to that outlined in Section 2.23.1. HRP was immobilised as described in Section 2.20. The dilution factor required was determined using the TMB assay (Section 2.2.1). 100μL of a 1:1000 and 1:100 dilution of the native and modified HRP respectively was added to the reaction mixture (Section 2.27.1) and the percentage of colour removed after 10 min at room temperature was determined (Section 2.27.1).

2.27.5 Effect of Successive Addition of Dye on Bleaching (Cycles of Bleaching)

8μM Chicago Sky Blue 6B, 50 μM Vanillin and 200 μM H_2O_2 in 100mM phosphate buffer, pH 7.0 were mixed with immobilised and free samples of HRP. The

bleaching effect was monitored over 10 min as the change in absorbance at 618 nm. The samples were then spiked with 20 µl of 400 µM Sky Blue and 50 µl of 2000 µM H_2O_2 and the bleaching effect monitored. Five cycles of bleaching was performed. In all instances reactions were carried out in triplicate and a control (the reaction mixture less HRP) was included.

2.28 Phenol Precipitation Reactions

Phenol precipitation reactions were carried out in triplicate in 10 ml vials. Reaction solutions were thermally equilibrated in a water bath (accuracy ± 0.5° C) at 37 °C or 70 °C. Mixtures were prepared by addition of 100µl 0.3U ml^{-1} HRP to a 1 mM phenol solution in 10 mM borate buffer, pH 9.0. Polymerisation was achieved by the addition of H_2O_2 (a final concentration 1 mM). Reaction mixtures were agitated in a shaking water bath. Reactions were terminated by the addition of catalase enzyme to a final concentration of 30 nmol dm^{-3}. The end point of the reaction was taken as 20 min. Samples were then treated with 40g L^{-1} alum [$Al_2(SO_4)_3.14H_2O$] to enhance colloidal particle coagulation. Reaction solutions were adjusted to pH 6.3 by dropwise addition of either hydrochloric acid or sodium hydroxide to optimise floc formation. pH in small reaction vessels was determined using Whatman pH indicator paper. The samples were allowed to stand at room temperature for 20 min after which time they were centrifuged at 2,000 rpm for 40 min at room temperature. Residual phenol, 4-chlorophenol and mixed concentrations were determined by direct spectrophotometric measurements of absorbance at 280 nm .Phenols had earlier been scanned to determine λ max. To determine the relative removal efficiency of this treatment, a solution of phenol is analysed before and after addition of the HRP system (HRP/H_2O_2). Peroxide, HRP, catalase and alum did not interfere with absorbance measurements at 280nm (Miland et al., 1996)

CHAPTER 3

STUDIES ON NATIVE HRP

3.0 Studies on Native HRP

3.1 Introduction

Horseradish peroxidase (HRP) is an oxidoreductase (donor: hydrogen peroxide oxidoreductase; E.C. 1.11.1.7; HRP). Like all peroxidases, it reduces hydrogen peroxide to water and oxidises a variety of substrates. (Welinder, 1992).

A wide range of indicator molecules exist, which are capable of assessing peroxidase activity. Assays for the detection of HRP activity towards hydrogen peroxide may exploit substrates that produce colorimetric, luminescent, fluorimetric or electrochemical reactions. The best assays provide a direct measure of enzyme activity, usually by coloured product formation (colorimetric assay). When colorimetric substrates are used as hydrogen donors, oxidation results in the formation of a coloured product. Chromogenic hydrogen donors for HRP include 3,3',5,5'-tetramethylbenzidine (TMB). This has been introduced as a non-carcinogenic alternative to benzidine, however, it does suffer from limited solubility in water (Liem et al, 1979). Generation of a blue colour when oxidised by the HRP/H_2O_2 complex indicates HRP activity. This change in colour can be monitored spectrophotometrically at 620 nm. Many other substrates have been quoted as qualitative and quantitative methods for detection of HRP. Another efficient substrate that is very often used is 2,2'-azino-di-(3-ethyl-benzothiazoline-(6)-sulphonic acid) or ABTS (Smith et al, 1993). This gives a characteristic green colour which can be followed spectrophotometrically at 405 nm.

The TMB assay was the assay of choice for most of the experiments carried out, as it was easily scaled down in order to utilise microtitre plates. This reduced the quantities of HRP used in a given experiment and enabled replicate assays to be carried out more easily.

Studies on a number of different characteristics of HRP were necessary prior to commencement of modifications. One of these was its thermal stability. The retention of stability and enzyme function is of vital importance. Investigations of thermophilic organisms, enzyme denaturation studies and the use of enzymes in organic solvents have each contributed to the understanding of enzyme stability. Enzymes can reliably and reproducibly be stabilised by a variety of means including immobilisation, use of additives,

chemical modification and protein engineering. (O Fagain and O'Kennedy, 1991). Some of these approaches will be discussed in detail.

Mozhaev (1993) explained the strategies for stabilisation in the context of a two-step model of irreversible inactivation which, he suggested, involved preliminary reversible unfolding followed by the irreversible step. Mozhaev suggested that the unfolding is best inhibited by covalent immobilisation, whereas other chemical methods, such as covalent modification of amino acid residues by adding low molecular weight compounds are very effective when "incorrect refolding" is the dominating irreversible process. The covalently linked polypeptide chains of proteins are folded in three dimensions in a pattern that is characteristic for that protein. The specific manner in which the chain is folded gives each protein its characteristic biological activity. Denatured protein can assume many different random coil formations that are usually biologically inactive. Denaturation was historically thought to be an irreversible process, e.g. egg white protein coagulated by cooking does not spontaneously go back into solution again on cooling. However, some proteins will regain their native structure if they are cooled slowly or returned to their normal environmental conditions (Creighton, 1993).

Klibanov & Mozhaev (1978) suggested a mechanism of irreversible denaturation of proteins/enzymes due to thermoinactivation. With an increase in temperature, protein molecules partially unfold and then may refold into new structures, different from the native enzyme conformation, to form kinetically or thermodynamically stable structures which are enzymatically inactive. Even after cooling, these incorrect structures remain because a high kinetic barrier prevents spontaneous refolding to the native structures. Thus, "irreversibly thermoinactivated enzyme" may be defined as an incorrectly refolded enzyme whose structure is supported by the same interactions: hydrophobic, electrostatic, hydrogen bonds, Van der Waals, - as the native conformation. However, due to intrinsic steric hindrance it cannot (during a reasonable experimental interval) transform into a correctly folded, catalytically active conformation. It has emerged in recent years that so-called "chaperones" may participate in an ordered sequence of events during protein folding. It has been well established for years that all the information for correct folding of the enzyme is contained within the amino acid sequence of the enzyme (i.e. information on

refolding is inherent in the amino acid sequence) and that kinetic control is also of importance where correct folding is concerned, (Ewbank & Creighton, 1992).

Exposure of the protein to high temperatures (usually temperatures in excess of 50° C) for a given period of time usually results in denaturation of the protein. However the exact temperature varies with the enzyme. For example, enzymes and other proteins isolated from thermophilic micro-organisms are known to be more stable than those isolated from mesophilic organisms living at more "normal" temperatures. (O'Fagain and O'Kennedy, 1991).

Prior to engaging in any modifications of the native enzyme it is important to establish a pattern of thermal denaturation and of protein stability. Having established these parameters it is then possible to make direct comparisons between the native enzyme and the modified form under similar conditions of denaturation. There are a number of sophisticated techniques available to follow the unfolding curves, including circular dichroism (CD) and fluorescence (Pappa and Cass, 1993). One can also measure the residual activity. Thermostability ($N \rightarrow I$, where N is the native form and I is the irreversibly inactivated form of the protein) can be measured in this way by incubating the sample at a particular temperature for a certain length of time and measuring its residual activity. This is a relatively simple and fast technique to perform. Various incubation temperatures were studied to ascertain a temperature at which the enzyme inactivation rate could be conveniently measured. One particular temperature was chosen (65 °C) and HRP's activity loss with time was studied to determine the reaction order of its activity loss.

The correct concentration range of HRP to work within was determined at room temperature (a number of the parameters had previously been determined by Ryan et al., (1994).

3.2 Results

3.2.1 Optimisation of the TMB Assay

The assay was performed as per Section 2.2.1., a concentration of 10μg L^{-1} was established as being within acceptable parameters (See Fig.3.1). The effect of varying the exposure time to the TMB was also investigated and 2 min was found to be the optimal assay time at which to determine the activity (See Fig. 3.2).

3.2.2 Thermal Stability of HRP

3.2.2.1 Effects of Varying Buffers

Thermoinactivation of HRP at various temperatures was performed as described in Section 2.10. At 65 °C the residual activity of HRP was typically less than 10% and at 75 °C there was a total loss of activity (Fig. 3.3). Thermoinactivation curves for native HRP incubated at 60 °C and prepared in 10mM phosphate buffer, pH 7.0 and in 10mM MOPS, ,pH 7.0 are shown in Fig. 3.4a. The curves are plotted as % residual activity vs time. An even rate of decay was noted for the samples over time

The kinetics of thermoinactivation of HRP in the two buffers at 60 °C were studied (Fig.3.4a, b, ,c). The results of % residual activity vs time plots were analysed using the computer programme Enzfitter. Data was fitted to the first order exponential decay equation and visual observation of the graphic fit shows that thermoinactivations obeys a first order rate equation. The standard deviations of the first-order rate constant, k, and the intercept were low, less than 5%. The half life for the native HRP prepared in MOPS buffer was more than twice that of the native HRP prepared in phosphate buffer and incubated at 60 °C (Table 3.1).

Fig. 3.1 HRP concentration versus absorbance

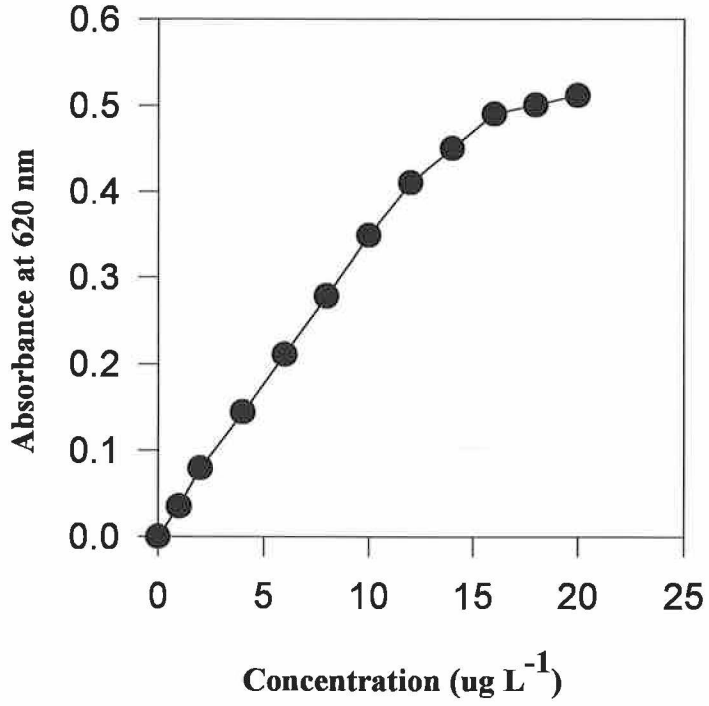


Fig. 3.2 Effect of assay time on TMB assay results

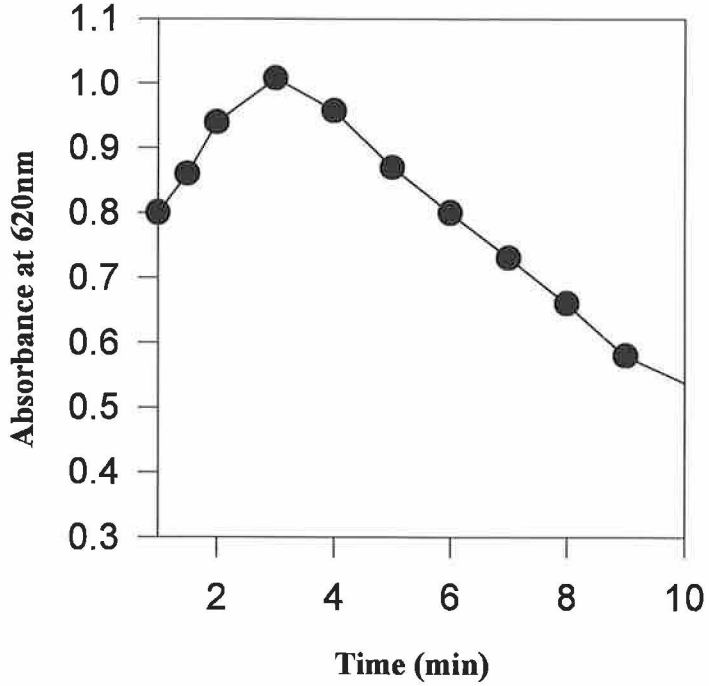


Table 3.1 Comparison of Native HRP in Phosphate and MOPS Buffer and Effects of $CaCl_2$ and EDTA

	Phosphate	MOPS	($CaCl_2$) Phosphate\MOPS	(EDTA) Phosphate\MOPS
k value	0.1 ± 0.004	0.04 ± 0.002	0.074± 0.003 \0.03 ± 0.001	0.098 ±.005 \0.02 ±.0003
$t_{1/2}$ (mins)	6.6	16.46	9.2 \ 29	7.04 \ 33

3.2.3 Effect of $CaCl_2$ and EDTA

The assay was performed in accordance with the method outlined in Section 2.11.b. The effect of incubation in the buffers outlined above, with 1mM $CaCl_2$ and 1mM EDTA were investigated. It was found that $CaCl_2$ exerted a protective effect on HRP prepared in phosphate buffer, increasing the half life from 6.6 (k=0.0042) to 9.2 min (k=0.0024). No significant change in half life was noted with the inclusion of EDTA. Almost a 2 fold increase in $t_{1/2}$ was noted for the addition of $CaCl_2$ and EDTA in the case of the MOPS buffer (Table 3.1).

3.2.4 Effects of Different Assay Substrates

The thermal stability of native HRP in 10mM, phosphate buffer, pH 7.0 was determined using the TMB assay (Section 2.2.1) and the ABTS assay (Section 2.2.2). The first assay involved storing the samples on ice after withdrawl from the water bath at set time intervals, while in the latter method the samples were assayed immediately. The difference in $t_{1/2}$ for the native HRP assayed using the two methods was negligible (Fig. 3.5)

Fig. 3.3 Thermostability studies on native HRP at different temperatures

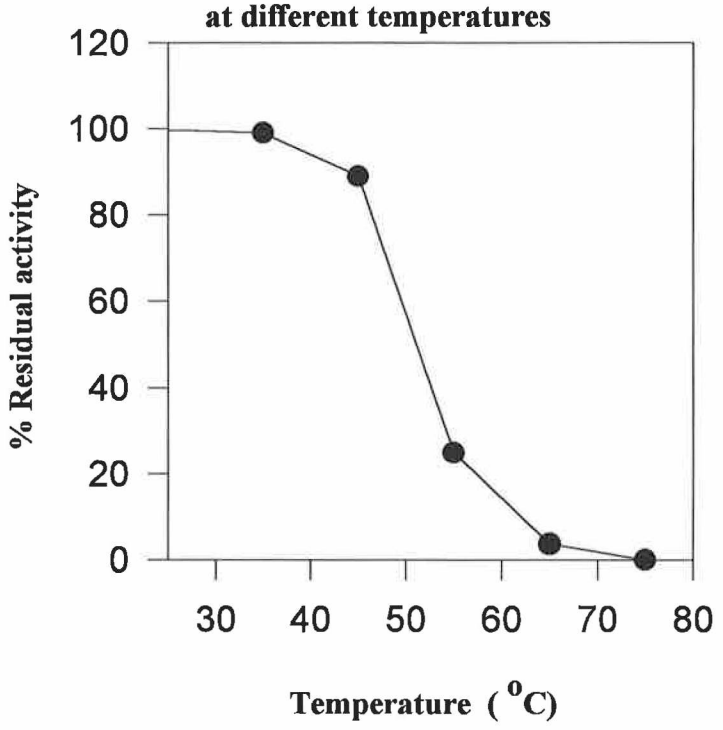


Fig. 3.4a Thermoinactivation studies on native HRP in phosphate and MOPS buffer

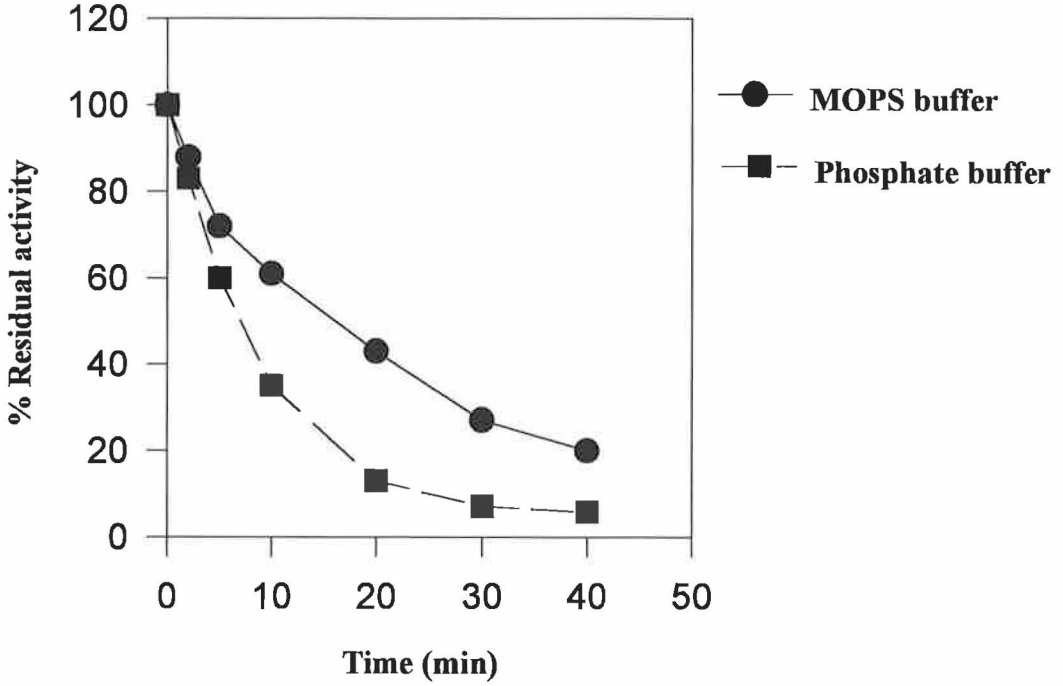


Fig. 3.4b

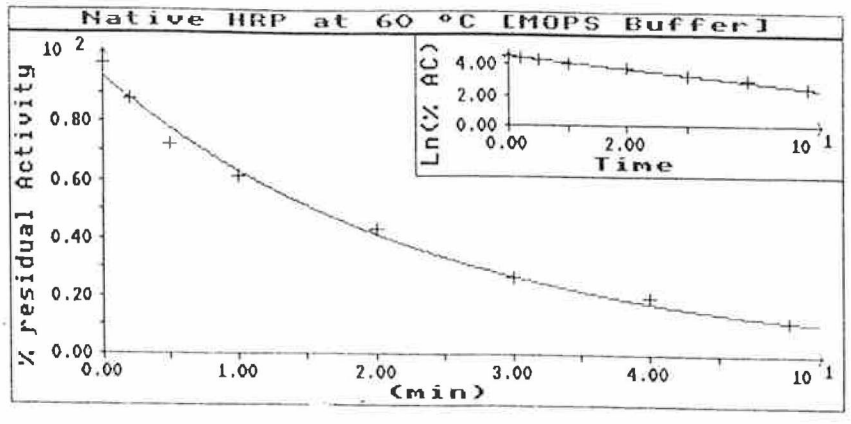


Fig. 3.4c

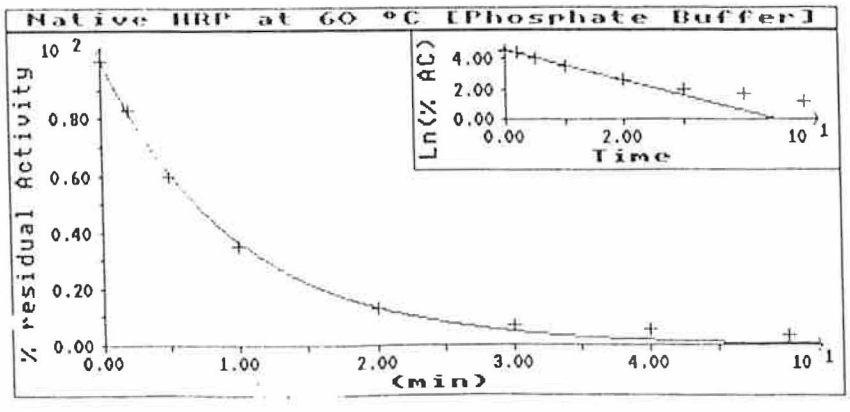
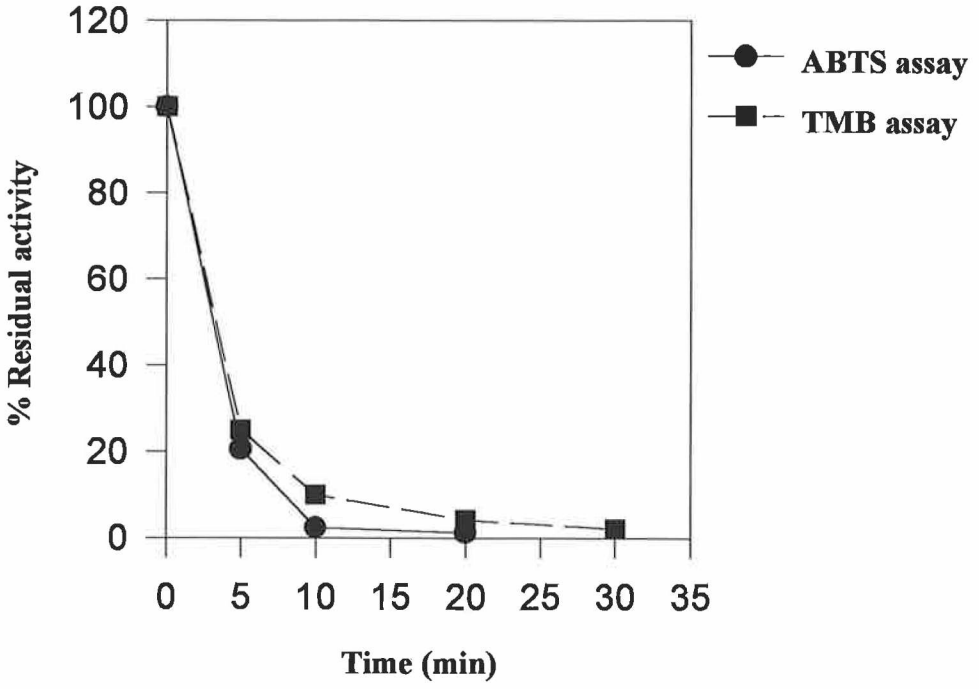


Fig. 3.4b and 3.4c. Single exponential decay curves with semi-log plots inset. These were plotted using the PC based package "Enzfitter"

Fig. 3.5 Thermoinactivation profile of native HRP at 65 ^{0}C



CHAPTER 4

IMMOBILISATION AND CHEMICAL MODIFICATION

4.0 Chemical Modification and Immobilisation

4.1 Introduction

It is well known that an enzyme in its native form is marginaly stable, and different factors can decrease its enzymatic activity which disturb the tertiary structure, (Mozhaev et al, 1993). One of the aims of chemical modification is to reduce the lability of enzymes, since enzymes with increased stability are often required for different applications, such as organic synthesis, chemical analysis, therapeutics and diagnostics, polymerisation of phenols, use in biosensors and in the many other diverse biotechnological transformations (Moreno & O' Fagain, 1996). The chemical modification of enzymes has been a valuable tool for investigating the nature of the active site residues. It can identify those amino acids that participate in catalysis and those that are important in substrate binding. Moreover, chemical modification of enzymes has been used to study reaction and substrate specificity, stability, cooperativity and the ionisation behaviour of functional groups. Through chemical modification it has been possible to change the pH optima of enzymes, the relative reactivity towards substrates and the pattern of substrate and product inhibition. Therefore, there is immense interest in chemically modified enzymes and the various modification techniques. Many methodologies have been developed to obtain stable proteins and some of these have been reviewed by O Fagain & O'Kennedy (1991).

Soluble enzymes can be chemically modified in a number of ways so as to alter their properties and a wide range of reagents for this purpose have been available for a number of years. Bifunctional reagents, useful for the preparation of conjugates for immunoassay, diagnostic imaging and other applications, also find wide use as enzyme stabilisers. These may be homobifunctional (with the same type of reactive group present at both ends of the molecule) or hetrobifunctional, (where there is a different reactive group present at either end of the molecule). Among the bifunctional groups widely used are bis-succinimides which react with amino groups to bring about crosslinking.(O' Fagain & O'Kennedy, 1991).

Chemical modifications are in many ways complementary to site directed mutagenesis and protein engineering as a methodology for the study of protein variants. The mechanisms of chemical stabilisation can be categorised under four headings

1. Crosslinking by bifunctional reagents

2. Strengthening of hydrophobic interactions by nonpolar reagents.
3. Introduction of new polar or charged groups leading to additional ionic or hydrogen bonds
4. hydrophilisation of the protein surface to reduce unfavourable surface hydrophobic contacts with water

An extensive table of side-chain functional groups and reagents for their modification is given in O' Fagain & O'Kennedy (1991).

A number of chemical modifications were carried out on HRP in order to ascertain the stability effects on the enzyme, arising from modification of the target residues by the various reagents. This was performed by the systematic modification of the various available amino acid residues of HRP.

4.1.1 Tryptophan Residue:

The single tryptophan residue of HRP is unavailable for modification, (Welinder 1979) as it is obscured by the haem component. Therefore, in order to carry out the modification, the haem would first have to be removed (creating an apo-enzyme). This would involve severe treatment of the enzyme which would probably render the HRP inactive and thereby negate any possible beneficial effect of the modification. In enzymes with more than one tryptophan N Bromosuccinimide (NBS) has been used. BNPS-Skatole and iodine can also be used (O' Fagain et al, 1991), this would result in cleavage of the tryptophanyl peptide bonds resulting in total enzyme inactivation. However, when the enzyme was incubated with its substrate or competitive inhibitor the loss of activity was greatly reduced. This shows that the essential tryptophan was being protected by the substrate/inhibitor. On removal of the inhibitor and retreatment with NBS, activity was completely lost with concomitant modification of the tryptophan residue. This indicates that only one tryptophan is at the active centre of α-Taka amylase and is essential for activity (Kochhar & Dua. 1985). Kita et al. (1982) also noted that 5 out of 9 tryptophan residues in Taka- amylase were modified with NBS. Only one of these was located at the binding site and its modification affected catalytic function. This evidence provides a further reason for not undertaking chemical modification of the single HRP tryptophan as that residue (Trp-117) is located in a hydrophobic environment devoid of proton accepting bases and also its position is remote from the catalytically active area (Ohlsson et al., 1986).

4.1.2 Other Amino Acid Residues

The carboxyl groups of glutamic and aspartic acid residues can be modified by carbodiimides, a reaction involving amidation of the carboxyl group (Hunter & Ludwig 1972). Ryan et al. (1994) undertook this modification and no observable effect was noted with regard to the stability of HRP.

Various electrophiles were employed by Urrutigoity et al. (1991a) to modify the histidine residues of HRP. They found that pyrocarbonates were particularly effective, and were able to use their chromatic effect to determine the number of histidines modified. They demonstrated that the catalytic activity of HRP was not affected by small substitutions lacking reactive groups. Urrutigoity et al, (1991b) later showed that these modifying reagents could be used to alter the active site of the HRP without dramatic loss of activity.

Methionine is reactive with H_2O_2 (O'Fagain & O'Kennedy, 1991), however, excessive H_2O_2 can seriously disrupt the enzyme and result in its inactivation.

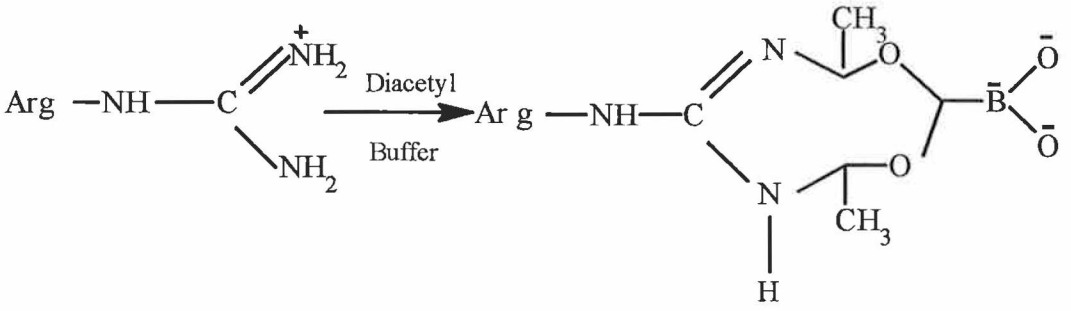
4.2 Modification of Arginine

4.2.1 Treatment with Diacetyl and Glyoxal

The guanidinium group of arginine (pK_a = 12.5) is a planar, resonance stabilised structure which can exist in its ionised form over the pH range 6.0-9.0 where it is relatively stable. This nitrogen function is known to have a marked resistance to acylation (and indeed to other common amine reactions), except under rigorous conditions which could prove very disruptive to the stability of the enzyme. This formerly prevented its detailed chemical investigation in proteins (Yankeelov, 1972). It was demonstrated by Fliss & Viswanatha (1979) and later by Gripon & Hofmann (1981) that arginine could be modified with 2,3-butanedione (diacetyl) and glyoxal at or near neutral pH without loss of enzyme activity. This left the way open for further studies into the various applications of the modification. The importance of arginine has long been known; in fact more enzymes are known to contain arginine than any other amino acid. This is because arginine generally serves as an anion binding site, particularly for phosphate and carboxylate groups. More than two thirds of all known enzymes either act on anionic substrates or require anionic coenzymes (Riordan, 1979).

Diacetyl is also known to be a potent photosensitiser and, therefore, photochemical side reactions may be involved in any arginine modifications. With this in mind the modification of HRP using this reagent was carried out both in the presence and absence of light. The possible mechanism of the reaction is shown schematically in Fig. 4.1. This reaction produces a change of 4 charges (from +1 to -3).The isoelectric point of the arginine-modified enzyme will be significantly reduced. While arginine does not appear to be involved in the catalytic cycle, enzyme activity is conserved possibly via a functional substitution by a neighbouring amino acid residue (Urrutigoity et al., 1993).

Fig. 4.1 Schematic of Possible Mechanism of Modification by Diacetyl



Glyoxal reacts with arginines at alkaline pH, notably around 9.2. The product is relatively stable . However, it appears to be somewhat less specific for arginine than is diacetyl. The structures of the two reagents can be seen in Fig 4.2 and 4.3.

Fig. 4.2 2,3 Butanedione (Diacetyl)

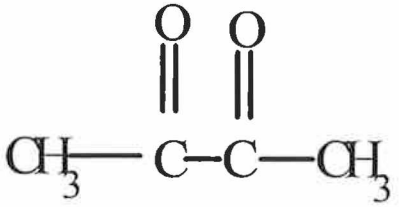
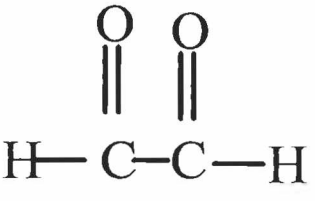


Fig. 4.3 Glyoxal



4.3 Nitration of Tyrosine Residues

4.3.1 Treatment with Tetranitromethane (TNM)

Tetranitromethane is a selective and mild reagent for the nitration of tyrosine and tyrosyl residues in peptides and proteins (Riordan et al., 1967). The product of the reaction in non-sulfhydryl proteins is an ionisable chromophore (3-nitrotyrosine). Therefore, nitration could be used to probe the immediate chemical environment of the nitration-susceptible tyrosyl residues which participate in the biological activities of proteins. It may prove possible to elucidate the chemical basis of the functional characteristics where nitration has taken place. Where the nitroprotein that results is an enzyme which maintains activity, then perturbation of the nitrotyrosyl spectrum through the binding of substrates or inhibitors might allow the exploration of the active centre.

4.4 Modification of Lysine Residues

4.4.1 Modification with Succinimides

N-Hydroxysuccinimide (NHS) esters are among the most popular of the modifying reagents. This is due largely to their commercial availability and mild chemical reaction conditions. The reaction of NHS esters occurs in the pH range 6.0-9.0 (Ji, 1983) yet, it takes place preferentially at the upper end of that pH range. Unfortunately, NHS-esters are not very soluble in aqueous buffer and need to be dissolved in a minimal volume of an organic solvent such as dimethyl sulphoxide (DMSO), prior to its addition to the enzyme in a buffer free from extraneous amines. Once added, it forms an emulsion and the subsequent reaction is rapid.

The NHS ester reaction consists of two competing reactions in aqueous solution. The reaction involves the nucleophilic attack of an amine on the acid carboxyl of an N-hydroxysuccinimide ester to form an amide (See Fig 4.4). Under normal reaction conditions lysyl residues are the second strongest nucleophiles in a protein molecule, cysteine being the most reactive; (Lundbland & Noyes, 1985). During the reaction, the positive charge of the amino group will be lost and N-hydroxysuccinimide released. Hydrolysis the competing reaction is favoured in dilute protein solutions and acylation in more concentrated solutions.

Most accessible amino acid residues will be attacked within 10-20 min. A 10-fold molar excess of an NHS ester will usually achieve acylation of the amino groups within a short time and within a wide temperature range (4-25°C); (Ji, 1983).

4.4.1.1 Crosslinking with N-Hydroxysuccinimide esters.

Bis-N-hydroxysuccinimide esters are homobifunctional crosslinking reagents. The crosslinking distance will have a considerable effect on the success of the crosslink formation. Reagents are available with various chain lengths. According to Ji (1983) chain lengths less than 5Å will yield few or no crosslinks.

Ethyleneglycol bis-N-hydroxysuccinimide ester was used in this reaction. The structure is shown in Fig. 4.5. The length of the molecular crosslink of the EG-NHS is 16Å (Ji, 1983). The EG-NHS reacts with primary amino groups mainly lysines, as they are strong nucleophiles. The modification procedure was carried out as outlined in Section 2.4.1, without further optimisation. The stabilised crosslinked derivatives of HRP were then characterised. The effects of this modification were compared to HRP modified by other reagents, i.e, comparison of crosslinking with covalent modification was undertaken.

Fig. 4.4 **NHS Ester Reaction**

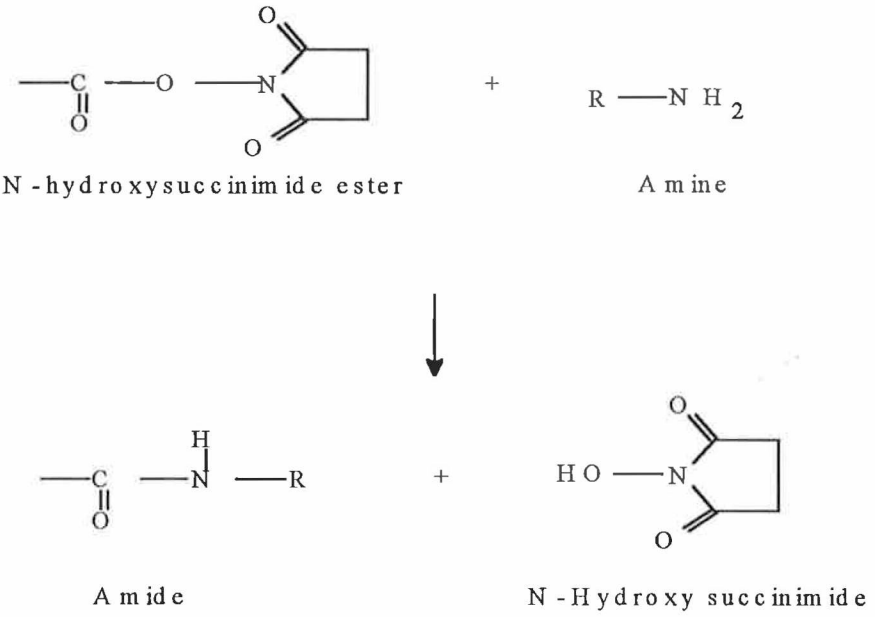
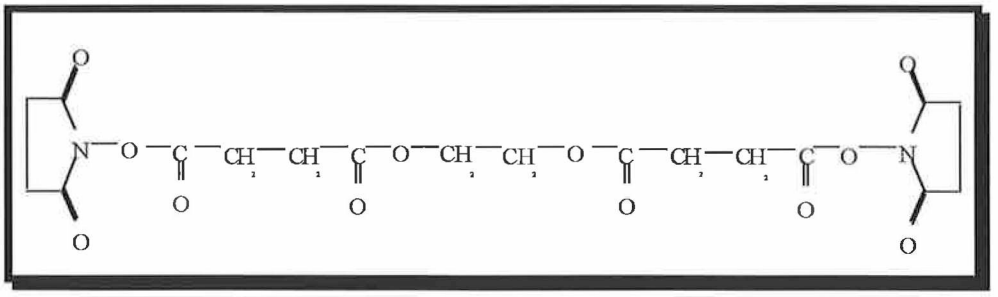


Fig.4.5 **Structure of Ethylene glycol-bis (succinic acid N-hydroxysuccinimide ester)**



4.4.1.2 Covalent Modification with Anhydrides

Cyclic anhydrides of aliphatic carboxylic acids (e.g. succinic and malic), as well as o-phthalic anhydrides are widely used as acylating reagents in protein biochemistry (Lundbland and Noyes, 1985). Phthalic anhydride is the principal commercial form of phthalic acid and has been used for many years as an acylating agent. Acylation of amino groups is a common means of chemical modification of enzymes. One of the reasons for this is that amino groups, being charged, tend to be located on the surface of an enzyme in contact with the ambient environment (Riordan, 1967). Non-polar residues are grouped together as hydrophobic surface clusters, however, lysine residues are charged groups. These play an important role *in vivo*, since they enable proteins to bind via hydrophobic interactions to other molecules e.g. other proteins, lipids, polysaccharides, substrates, etc. Since contact of nonpolar residues with water is thermodynamically disadvantageous and is harmful for protein stability *in vitro,* then a reduction of the nonpolar surface area should stabilise the protein (Mozhaev et al, 1988).

For the acylation of amino acid groups of HRP, a series of aromatic carboxylic acid anhydrides were used. These included a) phthalic anhydride, b) trimellitic anhydride, c) pyromellitic anhydride. These replace the positive charge of lysine with one, two and three negative charges respectively. There structures are displayed in Fig 4.6, Fig 4.7. and Fig 4.8 respectively. These reagents are insoluble in aqueous buffer and were first dissolved in a suitable organic solvent such as DMSO. The pH of the reaction mixture was kept constant through out the experiment while maintaining the temperature at 4 °C.

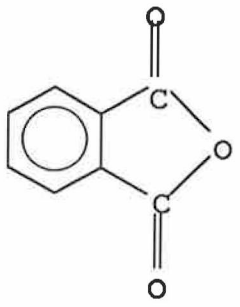


Fig. 4.6 Phthalic anhydride

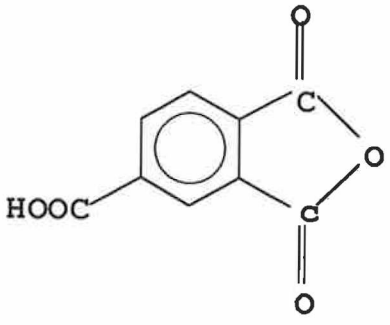


Fig. 4.7 Trimellitic anhydride

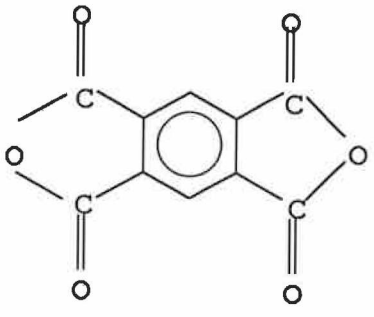


Fig. 4.8 Pyromellitic anhydride

Fig 4.6, 4.7 and 4.8 Structure of Aromatic Anhydrides

4.5 RESULTS

4.5.1 Modification of Arginine by Treatment with Diacetyl and Glyoxal

4.5.1.1 Optimisation of Reaction

The modification reaction was optimised with respect to diacetyl and glyoxal concentration. The following concentrations 15mM, 20mM, 25mM diacetyl and 10mM, 20mM, 25mM glyoxal were included in the reaction mixture. The recovered activity of all the derivatives was less than the native in all cases (Table 4.1). In all cases, the % activity recovered decreased with increasing concentrations of modifying reagents. 15mM and 10mM diacetyl and glyoxal respectively retained the highest activities. The reaction was carried out at pH 7.0 and this pH was monitored throughout the incubation period. The pH change that occurred during the reaction was negligible.

The HRP diacetyl/glyoxal preparations were incubated in the presence and absence of light. The light reactions consisted of incubating the samples for the reaction period in the fume hood with the light on (a 60W bulb at a distance of 1 metre) and subsequent dialysis was also carried out in the presence of light. The dark samples were covered in tin foil to exclude any light during the dialysis. However in the case of both modifying reagents, the presence or absence of light had no great effect on the samples.

4.5.1.2 Effects of Temperature on Diacetyl and Glyoxal Modified HRP

Thermostability of the two modified forms of HRP were compared with that of the native as described in Section 2.8.2. There was a slight modification of the method in this case as the temperature used was 62.5 °C as opposed to 65 °C. The modified forms did not show any increase in thermostability compared with the native. The half life of the native was 8 min whereas the diacetyl and glyoxal both had half lives of 5 min. (Fig 4.1 and 4.2).

4.5.1.3 Stability of Diacetyl and Glyoxal Modified HRP in Organic Solvents

Organotolerance was determined as described in Section 2.9. Organotolerance of native and the two modified forms of HRP were compared in DMF and THF at room temperature for 1 hour. At low concentrations of DMF the activity of the

modified HRP was enhanced and it remained active up to 40% DMF. There was slightly enhanced organotolerance compared with the native. No increase in organotolerance was noted in the THF.

TABLE 4.1 % Residual Activity Remaining After Modification of various Amino Acid Residues in HRP.

RESIDUE	MODIFIER	% CATALYTIC ACTIVITY
Lysine	Phthalic Anhydride	94%
	Trimellitic Anhydride	80%
	Pyromellitic dianhydride	80%
	EG-NHS	95%
Arginine	Diacetyl	87%
	Glyoxal	78%
Tyrosine	TNM	104%

TNM= Tetranitromethane,
EG-NHS= Ethylene Glycol-bis(succinic acid N-Hydroxysuccinimide ester)

4.5.2 Nitration of Tyrosine Residues with Tetranitromethane (TNM)

4.5.2.1 Optimisation of the reaction

Modification of HRP with the nitration reagent TNM was performed as per Section 2.4.4 . The activity of the native and the TNM HRP was determined using the TMB assay (Section 2..2.1). The reaction was optimised with respect to TNM concentration. The following molar excesses of TNM were included in the reaction mixture: 1.0, 10.0, 200.0 and 2000.0-fold and the resulting derivatives were analysed. It was found that the activity remained the same (97% to 104%) within the range 10 to 2000 fold excess; the 1:1 ratio gave the lowest % recovery (74%). The exposure time was also varied, the HRP being incubated with the reagent from 30 min to 120 min. It was found that the % activity recovered decreased after 1 hour incubation at room temperature (Table 4.2).

Fig. 4.1 Themoinactivation of native and diacetyl/glyoxal HRP at 62.5 °C in the presence of light

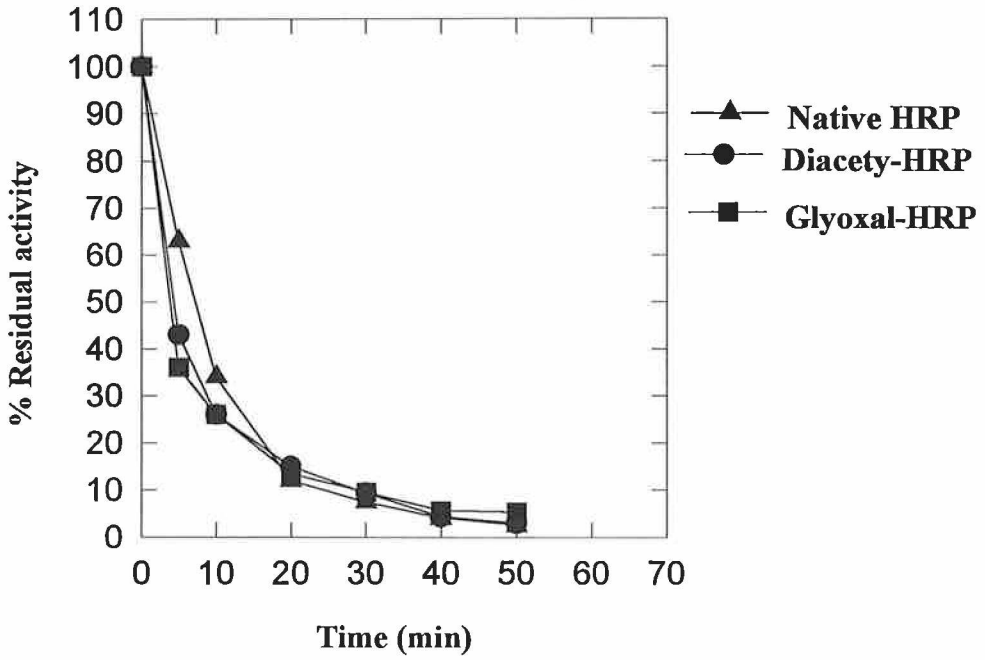
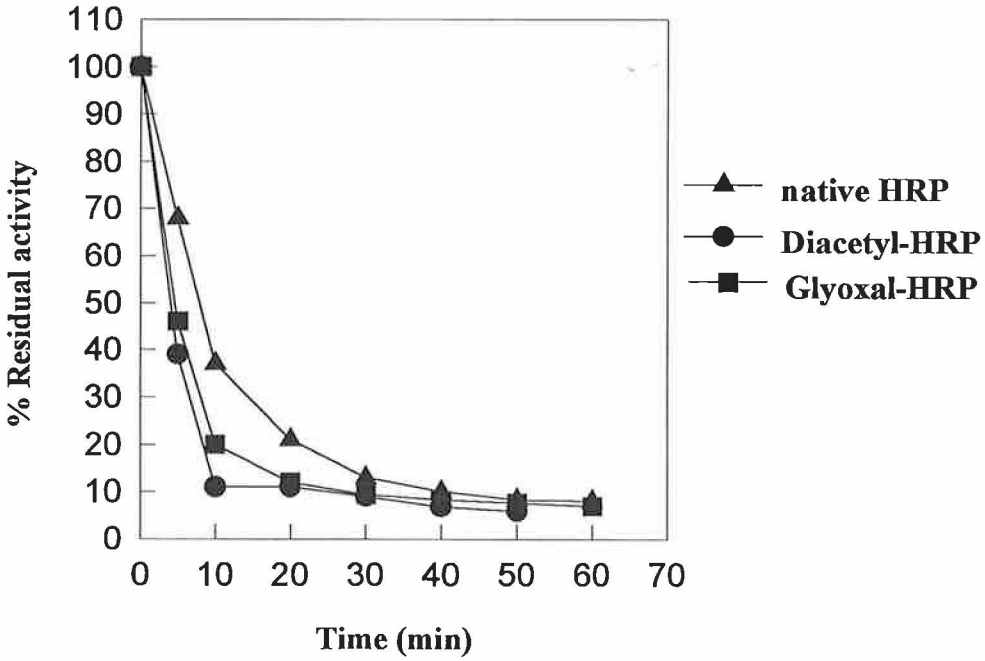


Fig.4.2 Thermoinactivation of native and diacetyl/glyoxal HRP at 62.5 °C in the absence of light



4.5.2.2 Effects of Temperature on TNM-HRP

Thermostability of TNM-HRP was compared with native HRP as described in section 2.8.2. TNM-HRP did not prove to be more thermostable than the native enzyme at 65 °C.

4.5.2.3 Stability of TNM-HRP in organic solvents

Organotolerance was determined as described in Section 2.9. Organotolerance of native and TNM-HRP were compared in methanol, DMSO, DMF and THF. The modified HRP showed no enhanced organotolerance. The C_{50} values were the same or less than the native in each solvent.

Table 4.2 % HRP Activities Retained by TNM-HRP Compared to Native With Respect to Exposure Time and Concentration of TNM.

Time (min) •	% Activity	molar Excess*	% Activity
30	96 ± 2	1:1	74 ± 1.2
60	97 ± 2.5	10	103 ± 1.41
90	75 ± 1.8	200	99.5 ± 3.5
120	70 ± 2.0	2000	97 ± 2.9

• At a molar excess of 200, * for 30 min incubation

4.5.3 Modification using Ethylene glycol-bis(N-hydroxysuccinimide) Ester

4.5.3.1 Optimisation of the Reaction

The reaction was optimised with respect to its termination. Initially an equal volume of Tris buffer had been used to stop the reaction; however, it was found that Sephadex G-25 gel filtration (Section 2.7) gave % recoveries in the same region as the Tris buffer method i.e. recovery in excess of 95%. Using this method, there was minimal dilution of the modified enzyme; This proved useful for a number of applications. The experimental procedures were carried out as per Section 2.4.1. The procedure was performed without

further changes as the method had previously been optimised by Miland et al. (1996). These conditions were successfully applied to the HRP.

4.5.3.2 Concentration of EG HRP

The concentration was determined by the BCA assay (Section 2.3.1) and checked spectrophotometrically as per Section 2.3.3. The sample was then diluted to the assay concentration of 10 $\mu g\ L^{-1}$ in 10 mM phosphate buffer pH 7.0 + 0.02% Tween 20.

4.5.3.3 Characterisation of the EG-NHS HRP

The % activity remaining after modification of HRP was determined using the TMB assay (Section 2.2.1). A table of the % activities remaining (% recovery) after each of the modifications was constructed (Table 4.3). In the case of the EG-NHS modifications recovery in excess of 90% was obtained in most cases.

From the free amino group determination using TNBS, the number of lysine residues modified was calculated (Section 2.5.1). It was found that 40% of the lysines remained unmodified suggesting that approximately 3 of the 5 available lysines were modified. The fluorimetric assay using fluorescamine (Section 2.5.2) was used to double check the results and the same number of modified lysines was determined. The results of these assays are outlined in Table 4.3 and 4.4 and are in agreement with results obtained by Miland et al. (1996).

4.5.3.4 Effects of Temperature on EG-NHS HRP

The thermostability of the EG-NHS HRP was compared to that of the native enzyme as described in Section 2.8.2. Modified HRP was more thermostable than the native (Fig.4.3). The half life of the enzyme modified at 65^0 C was much greater than that of the native: 34 min ($k=0.004\ min^{-1}$) for the modified enzyme compared to 3 min ($k=0.018\ min^{-1}$) for the native enzyme. This 11-fold stabilisation effect at 65°C is possibly due to the effect of crosslinking lysine residues, which braces the protein backbone and thereby protects the enzyme against unfolding. The effects of different buffer systems were also investigated and the results are shown in Section 3.2.2.1.

The temperature profiles of native and modified HRP were determined as described in Section 2.8.1. The residual activity of the modified enzyme was higher compared with the native over the temperature range 25°C to 75°C (see Fig.4.4). Modified HRP

Fig. 4.3 **Thermostability studies on native and EG-NHS crosslinked HRP at 65 °C**

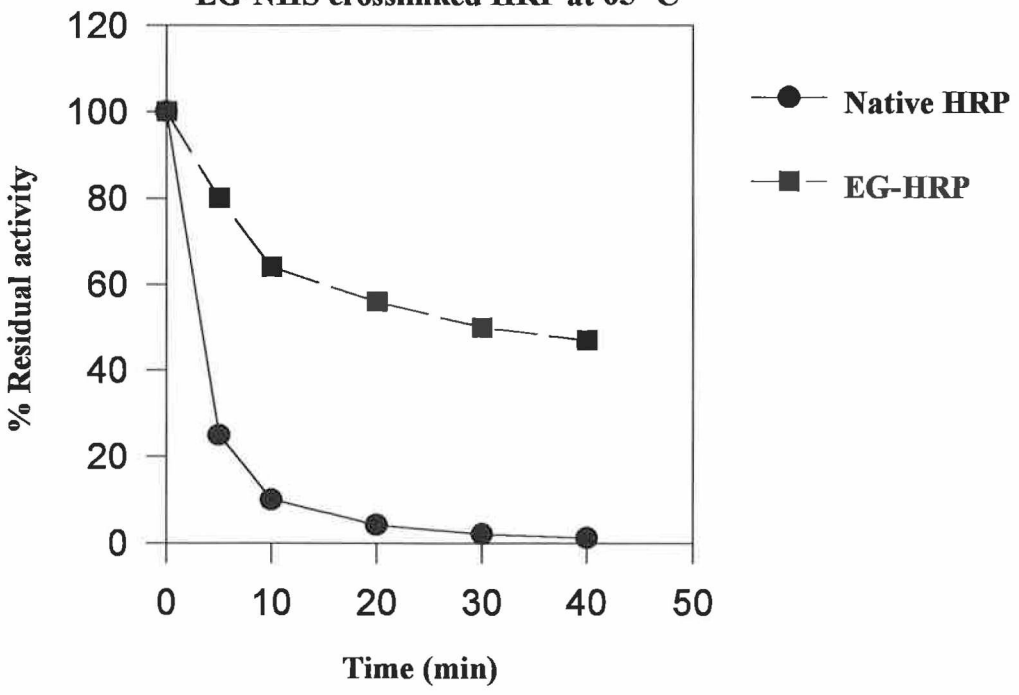


Fig. 4.4 **Temperature profile of native and EG-NHS modified HRP**

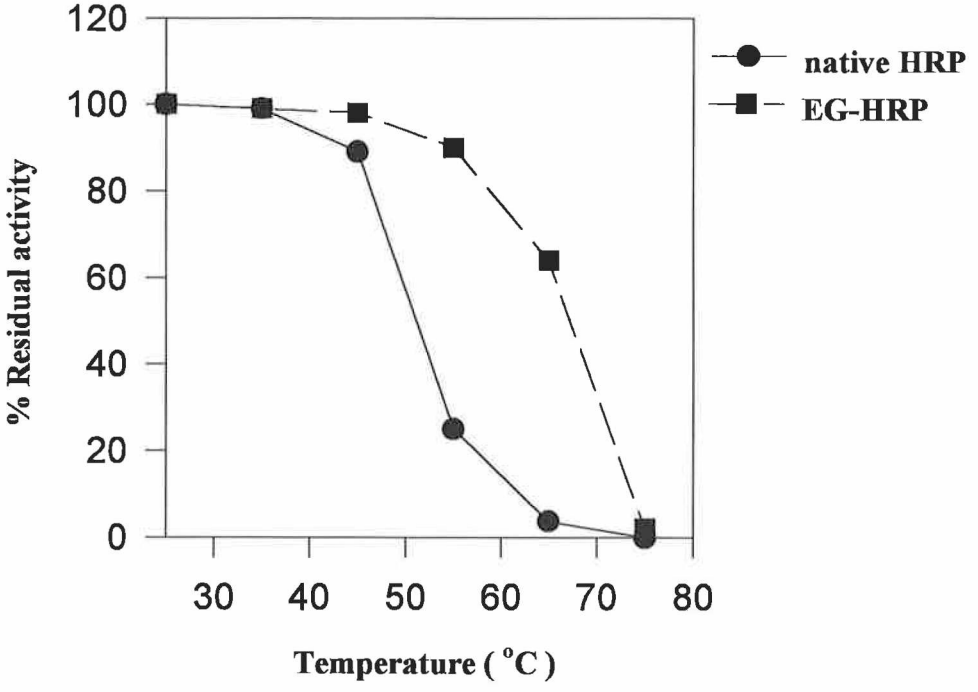


Fig. 4.5 Effects of DMF on native and EG-NHS HRP at room temp.

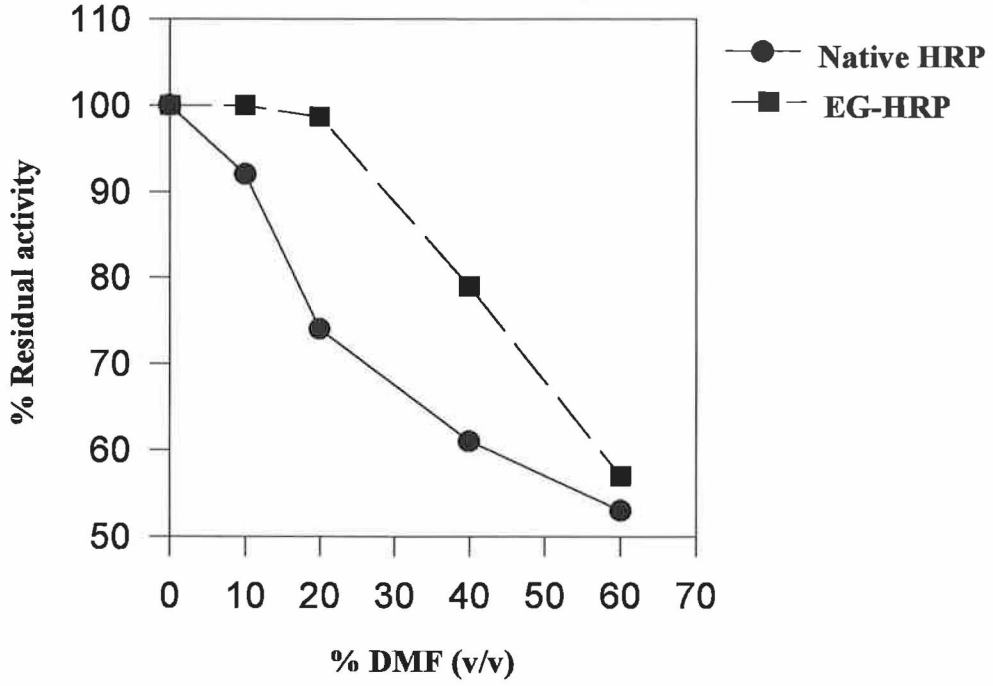
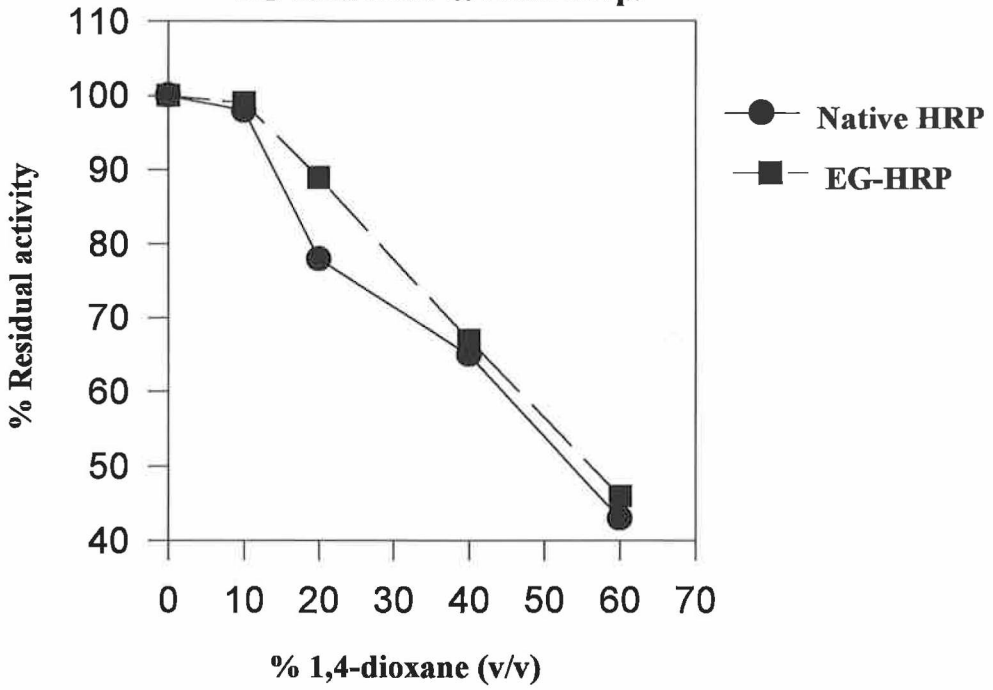


Fig. 4.6 Effects of 1,4-dioxane on native and EG-NHS HRP at room temp.



retained 90% activity up to 55 °C while the native enzyme lost some activity between 35 °C and 45 °C. T_{50} values for the native and modified were 49 °C and 63 °C respectively.

4.5.3.5 Stability of EG-NHS HRP in Organic Solvents

Organtolerances were determined as described in Section 2.9. The stability of both the native and the EG-NHS HRP were compared in DMF (Fig. 4.5), THF, DMSO, and 1,4-dioxane (Fig. 4.6). After incubation for 1 hour at room temperature the modified enzyme showed enhanced stability towards the solvents tested. At a concentration of 30% v/v 1,4-dioxane the modified HRP exhibited an 8% increase in stability compared to the native (Fig. 4.6)

4.5.4 Covalent modification with Aromatic Anhydrides

4.5.4.1 Optimisation of the Reaction

The modification of HRP by covalent modification with the cyclic anhydrides was carried out according to Section 2.4.2. The reagents used were phthalic anhydride, pyromellitic anhydride and trimellitic anhydride. The concentrations of the native and modified forms were determined using the BCA assay (Section 2.3.1). The % residual activity retained after the modifications was in excess of 90% for the phthalic anhydride while both other modified forms retained 80% (see Table 4.1).

Originally the samples were extensively dialysed against 10mM phosphate buffer, pH 7.0, to terminate the reaction and to remove any excess reagent prior to thermoinactivation at 65 °C. However G -25 gel filtration proved to be as effective and, as this was a much faster technique, was adopted as the method of choice.

4.5.4.2 Characterisation of PA-HRP

The PA-HRP demonstrated very good activity after the modification procedure (94%) compared with the native (see Table 4.1). From the free amino group determination using TNBS, it was found that approximately 60% of the lysines remained

unmodified, suggesting that 2 of the 6 lysines residues per HRP molecule were modified. The fluorescence assay using fluorescamine (Section 2.5.2) was used to double check the TNBS results The same number of lysines as determined to be modified with this method (see Table 4.3 and 4.4).

TABLE 4.3. Determination of free amino groups

(a) TNBS Assay

	NATIVE	EG-NHS HRP	PA-HRP
O.D.	0.84 ± .031	0.291 ± 0.017	0.549± .021
% UNMODIFIED	100%	40%	60%
NO. LYSINES MOD.	0	3/5	2/5

Table 4.4

(B) Fluorescence assay

	NATIVE	EG-NHS HRP	PA-HRP
FLUORESCENCE UNITS	25.87 ± 1.4	7.23 ± 0.41	19.21 ± 0.98
% UNMODIFIED	100%	40%	65%
NO.LYSINES MOD.	0	3/5	2/5

4.5.4.3 Effects of Temperature on HRP Modified with Anhydrides

Thermostability of the three anhydride modified forms was compared with the native as described in Section 2.8.2. Both pyromellitic and trimellitic anhydride modified HRP exhibited slight enhanced thermostability when compared with native enzyme. However, the phthalic anhydride (PA) HRP was more stable than the native at 65 °C (Fig 4.7). Because of the relatively low activity of the pyromellitic and the trimellitic anhydride derivatives and the absence of enhanced thermostability at 65 °C it was decided not to continue with characterisation of these forms and to concentrate instead on the PA-HRP. The half life for PA-HRP at 65 °C was 15 min ($k=0.004\ min^{-1}$) compared to 3 min ($k=0.018\ min^{-1}$) for the native HRP. Therefore, a 5 fold stabilisation was achieved. The residual activity of the PA-HRP was compared with the native over the temperature range 25 °C to 75 °C as described in Section 2.8.1. The PA-HRP retained 88% activity at 55 °C while the native retained just above 20% activity. T_{50} values for the native and PA-HRP were 51°C and 65 °C respectively, (Fig. 4.8).

4.5.4.4 Stability of PA-HRP in organic solvents

Organotolerance studies were carried out for PA HRP according to method outlined in Section 2.9. Organotolerance of the native and PA-HRP were compared in DMF, methanol, THF and 1,4-dioxane (see Fig.4.9-4.11 for dioxane, THF and DMF respectively) at room temperature. In most of the solvents tested PA-HRP demonstrated enhanced organotolerance. When PA-HRP was incubated at 65 °C for 10 min with THF, the degree of tolerance noted was more apparent than for solvent at room temperature (Fig. 4.12).

4.5.4.5 Effects of pH on PA HRP

pH profiles were determined as described in Section 2.11. The activities of PA modified and native HRP were compared over a range of pH values. The pH profiles for both were similar (Fig. 4.14). The pH profile for EG-HRP had been performed previously (Miland et al., 1996) and similar results were obtained. The activity of the modified and native HRP are maintained over a wide pH range.

Fig. 4.7 **Thermoinactivation of native and cyclic anhydride derivatives of HRP at 65 °C**

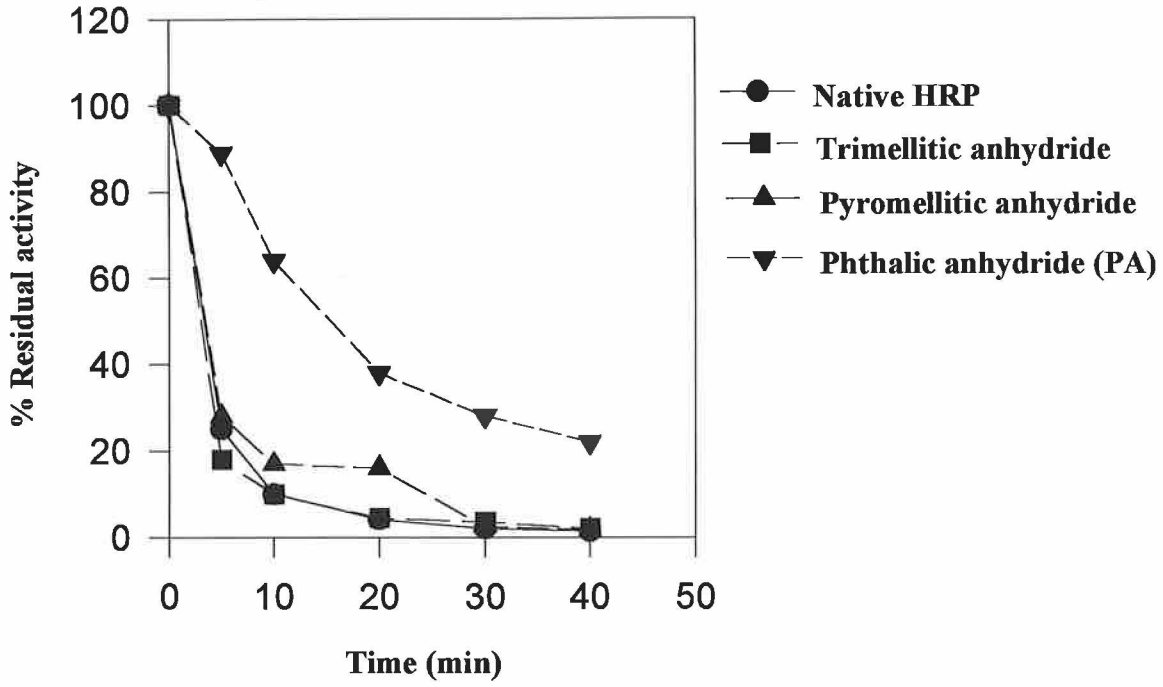


Fig. 4.8 **Temperature profile of native and PA-modified HRP**

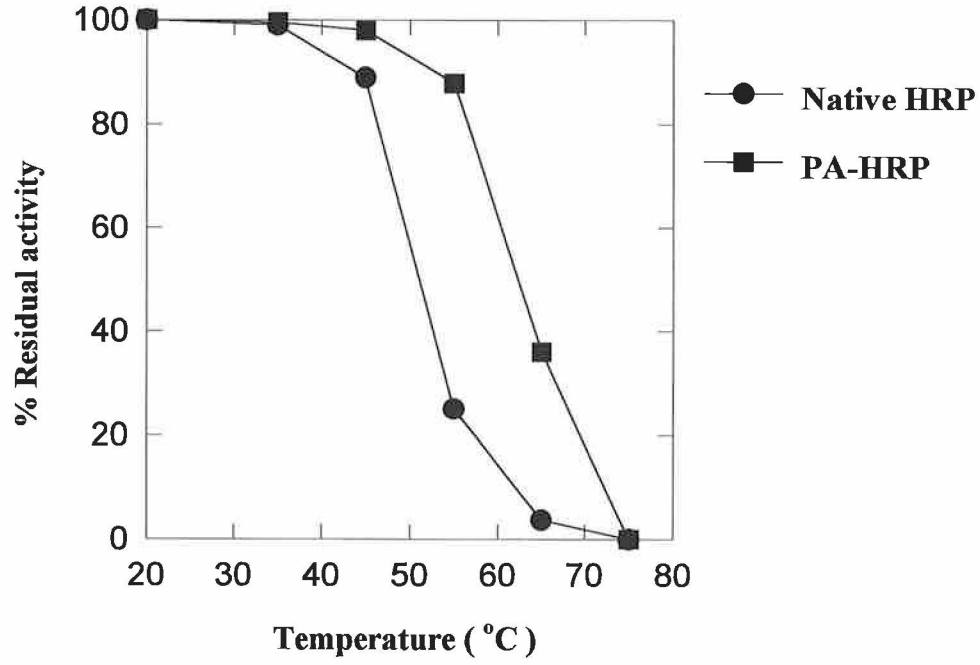


Fig. 4.9 Effects of 1,4-dioxane on native HRP and PA-HRP at room temperature

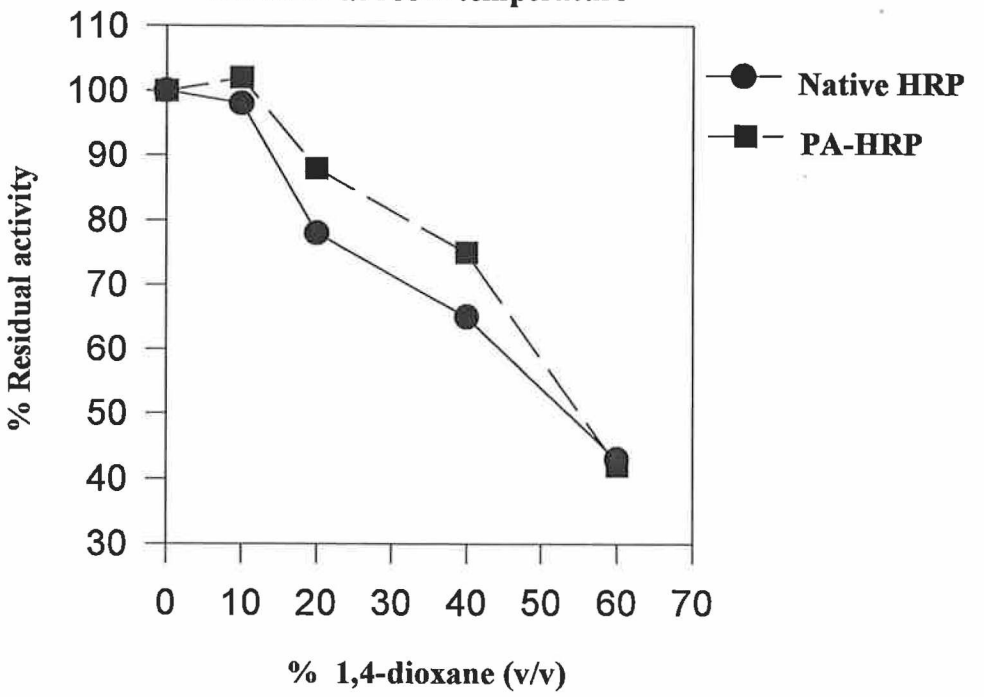


Fig. 4.10 Effect of THF on native and PA-HRP at room temperature

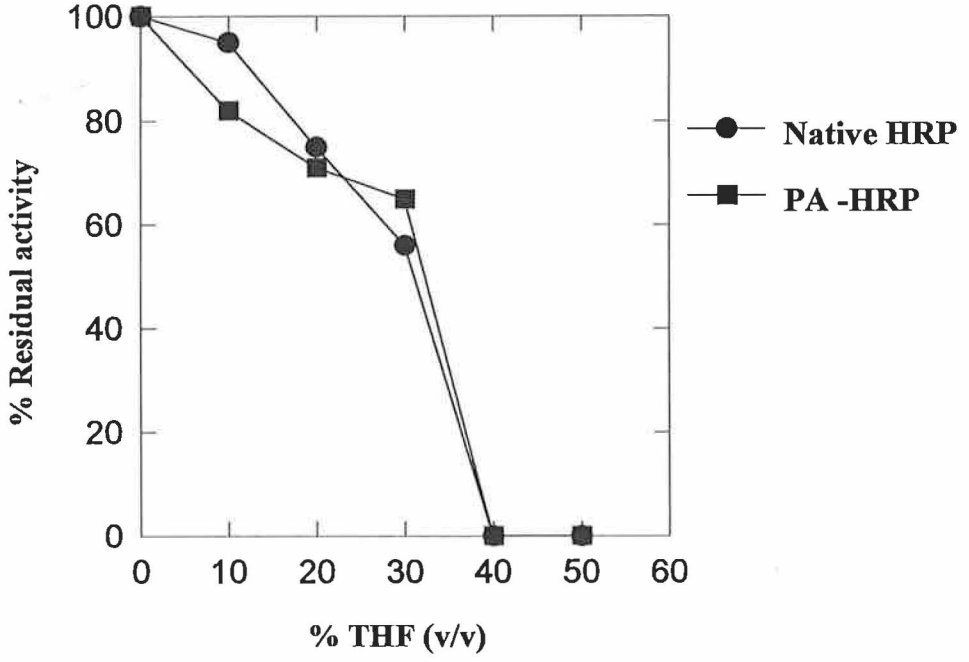


Fig. 4.11 **Effect of DMF on native and PA-HRP at room temperature**

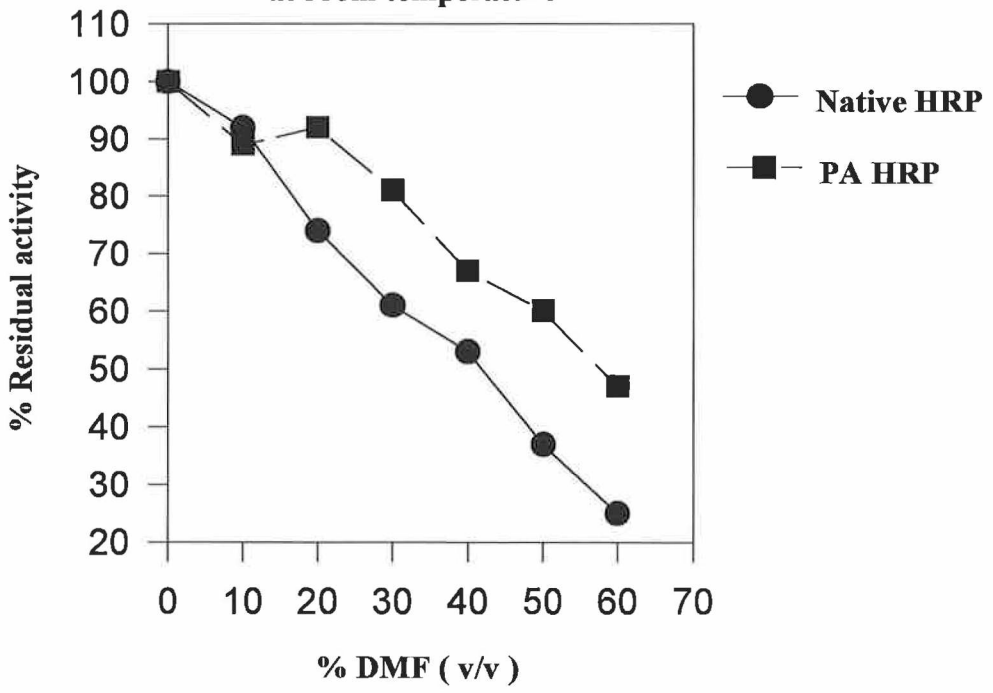
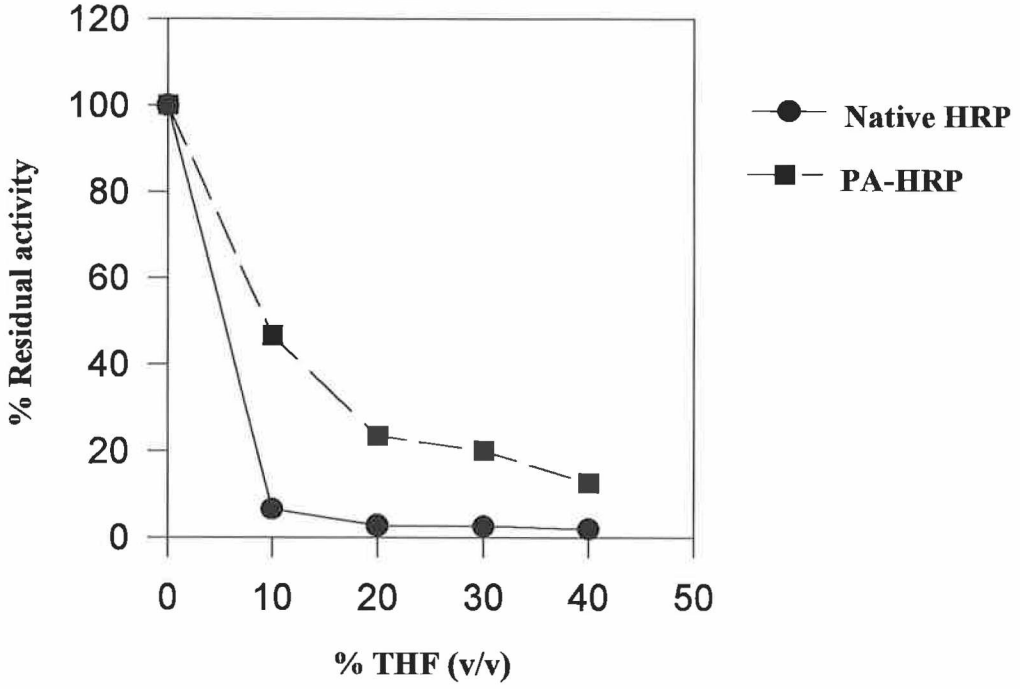


Fig. 4.12 **Effect of THF on native and PA-HRP for 10 min at 65 °C**



4.6 Hydrogen Peroxide Stability

Hydrogen peroxide stability is measured as the irreversible inactivation of HRP compound III formed in excess of hydrogen peroxide (Fig. 1.2). In the absence of a reductant substrate and with excess H_2O_2, native peroxidase yields firstly the active intermediate enzymatic form called Compound I. Operation through the two following pathways is possible: (a) a weak catalase reaction. Hydrogen peroxide can be used, in this case, as a reductant instead of an oxidant and (b) formation of Compound III from the reaction of Compound II with excess of H_2O_2 (Nakajima & Yamazaki, 1987).

A full kinetic study on the suicide inactivation of HRP by hydrogen peroxide was not carried out in this instance. The inactivation kinetics for the native HRP have been studied in detail by Arnao et al (1990). They established a value for the inactivation constant, k_i, and showed that both the catalase activity of the enzyme and the protective role of Compound III against H_2O_2-dependent peroxidase inactivation are important.

4.6.1 Hydrogen Peroxide Stability

The stability studies were carried out as described in Section 2.19. The half lives of the native, EG-NHS and PA HRP were determined. The decay curves did not follow first order kinetics. The $t_{1/2}$ (min) for the native, EG and PA HRP were 39, 38 and 33 min respectively. Fig.4.13 shows a scan of the native HRP over time on incubation with hydrogen peroxide.

4.7 Enzyme Immobilisation

A fundamental step in many biotechnology processes is the immobilisation of enzymes and proteins onto insoluble matrices. An immobilised enzyme is, by definition, one that is physically localised in a certain position or converted from a water soluble mobile state to a water insoluble one (Gianfreda & Scarfi, 1991). The thermal kinetics of immobilised enzymes have been shown to be different to their soluble counterparts. Immobilised enzymes provide us with a good model system to study as they simulate the state of the intracellular microenvironment of living cells. Information on enzyme reactions and intracellular microenvironmental properties has been obtained through the use of immobilised enzymes.

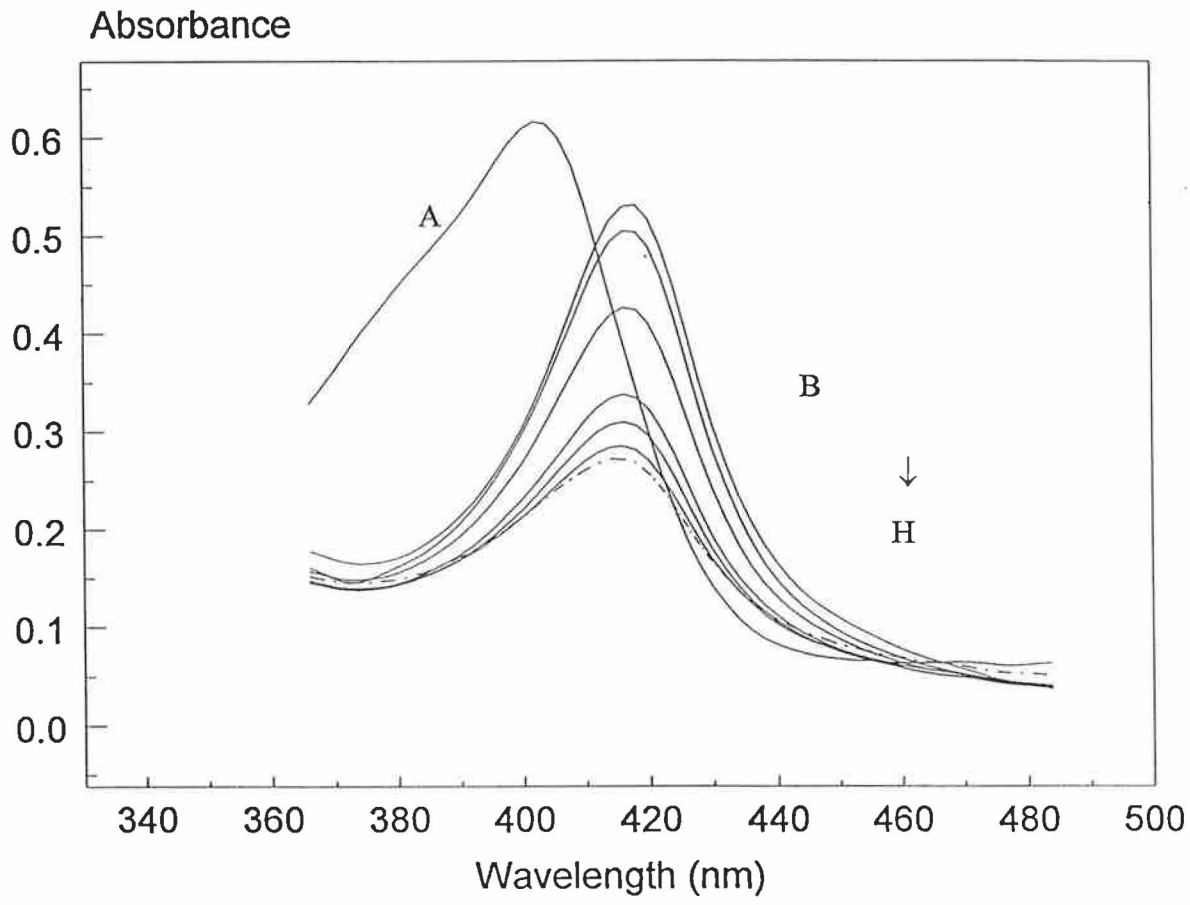


Fig. 4.13 Spectra obtained on titrating HRP with hydrogen peroxide over time in 100mM phosphate buffer, pH 7.0. The reference cell contained 10mM phosphate buffer, pH 7.0. The sample cell contained 5.8μM HRP in 10mM phosphate buffer, pH 7.0. and 4.4mM hydrogen peroxide at A) T 0, B) T 10, C) T15, D) T20, E) T25, F) T30, G) T35 H) T40.

There are four principal methods available for immobilising enzymes;

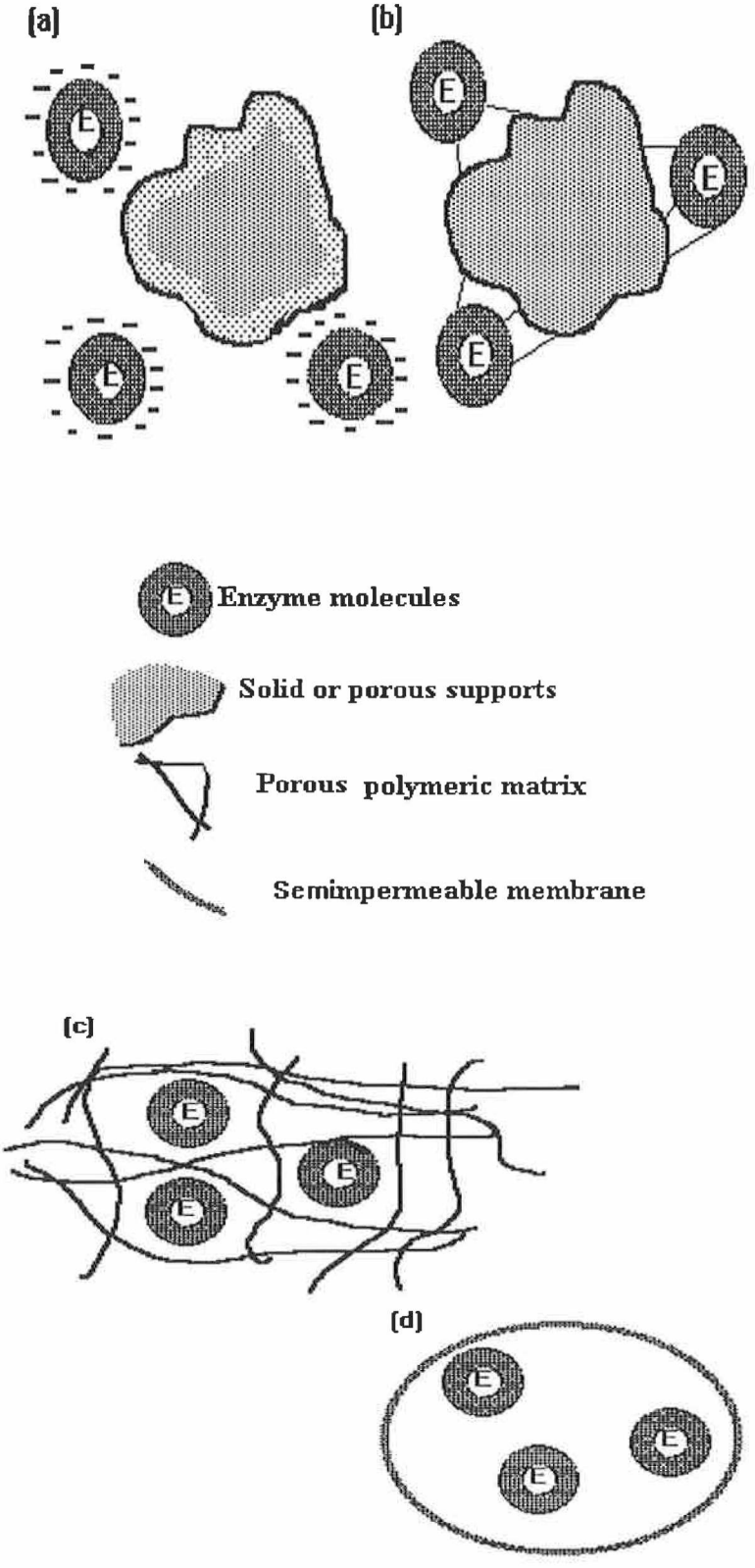
a) Adsorption,

b) Covalent binding,

c) Entrapment,

d) Membrane confinement.

Carrier matrices for enzyme immobilisation must be chosen with care, particularly where their use in industrial process is concerned. Cost is also a consideration. Ideally they should be cheap enough to discard following use. The nature of the support used can have a considerable effect on an enzyme's expressed activity and apparent kinetics. The ideal support is cheap, inert, physically strong and stable. It will increase the enzyme specificity (k_{cat}/K_m) whilst reducing product inhibition (Chaplin & Bucke, 1990). Scheme 4.1 illustrates the four principal methods of immobilisation. Immobilisation techniques have been assessed on native HRP and have proved very successful (Tor et al., 1989). As long ago as 1977, Martinek et al. noted an increase in thermostability of seven hundred-fold in derivatives of chymotrypsin and trypsin in the temperature range 60-100 °C. It was deduced that the greater the number of attachment points between the enzyme and the polymer support, the greater the degree of stabilisation achieved. While entrapment in calcium alginate beads has been shown to enhance stability in some molecules it is not suitable in every situation i.e. small enzymes will leach out over a period of time. Scouten, (1987) gives a detailed table of immobilisation techniques and supports used.



Scheme 4.1; Immobilised enzyme systems. (a) Enzyme non-covalently adsorbed to an insoluble particle; (b) Enzyme covalently attached to an insoluble particle; (c) Enzyme entrapped within an insoluble particle by a cross-linked polymer; (d) Enzyme confined within a semipermeable membrane

4.7.1 Thermostability of Immobilised Native and Modified forms of HRP

The HRP was immobilised as described in section 2.20. It was found that approximately 70% of the HRP was immobilised. Concentrations of immobilised HRP were found to be 1mg ml^{-1}, using the BCA assay (Section 2.3.1.1). The TMB assay (Section 2.2.1) was used to assay for relative activity and it was found that 1:1000 and 1:100 dilution's of immobilised native, EG and PA-HRP respectively, gave absorbance values that corresponded to approximately 5μg L^{-1} when compared with unimmobilised HRP assayed under the same conditions.

Thermostabilities of the immobilised HRPs were estimated as described in Section 2.8.2. Both of the modified forms demonstrated enhanced stability compared with immobilised native enzyme. The half life of the modified forms were almost 3-fold greater than the native HRP at 65 °C. The $t_{1/2}$ for the native , EG and PA were 9.0 min (k=0.01), 22 min (k=0.002) and 26 min (k=0.003) respectively. The thermostabilities of these immobilised forms were compared with the thermostability profiles carried out for the unimmobilised forms. It was found that native and PA-HRP were 3.5- and 2-fold more stable than their free counterparts respectively. The EG form whilst more stable than the native immobilised form, did not exhibit any enhanced stability when compared with unimmobilised EG-HRP. The thermoinactivation profile for the immobilised forms is shown in Fig 4.15.

4.8 Immobilisation in Calcium Alginate

The HRP was immobilised in calcium alginate as described in Section 2.20.1. The production of the beads was very successful and it was possible to produce large quantities of beads in a relatively short period. They were also easy to handle and did not disintegrate on drying. However, due to the relatively large pore size they did not prove successful as a means of immobilisation. When the beads were suspended in a solution the HRP leaked out. On the addition of hydrogen peroxide to the system this leakage could be clearly seen as the emergence of a blue stream. For this reason the immobilisation procedure was no more effective that using free HRP. In fact it was worse as the HRP was not distributed evenly in the solution.

The alginate beads were sealed in an air proof container and were assayed for activity 6 months later. It was found that 70% residual activity remained.

Fig. 4.14 **Effect of pH on Native and Modified HRP**

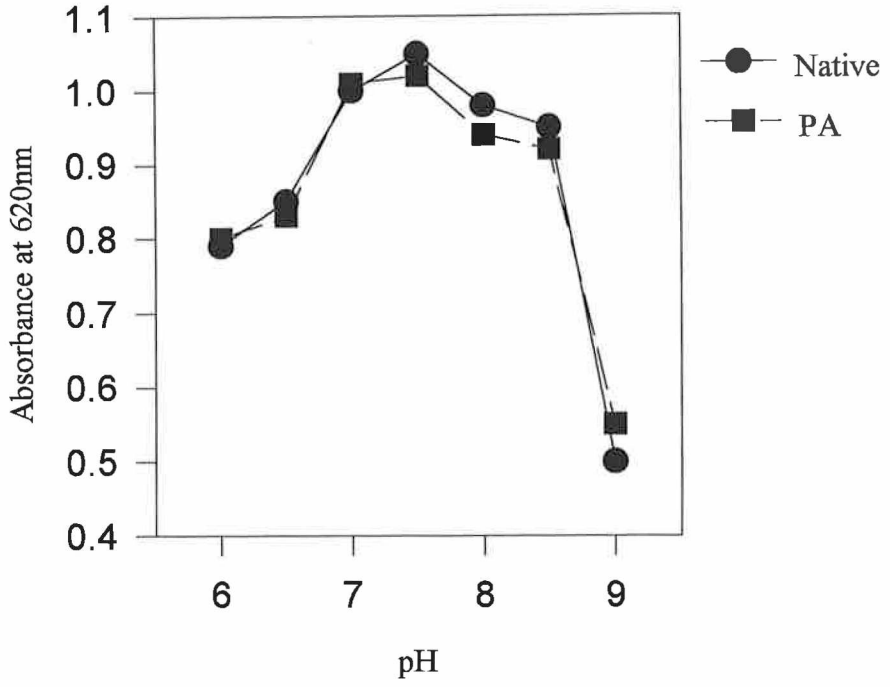
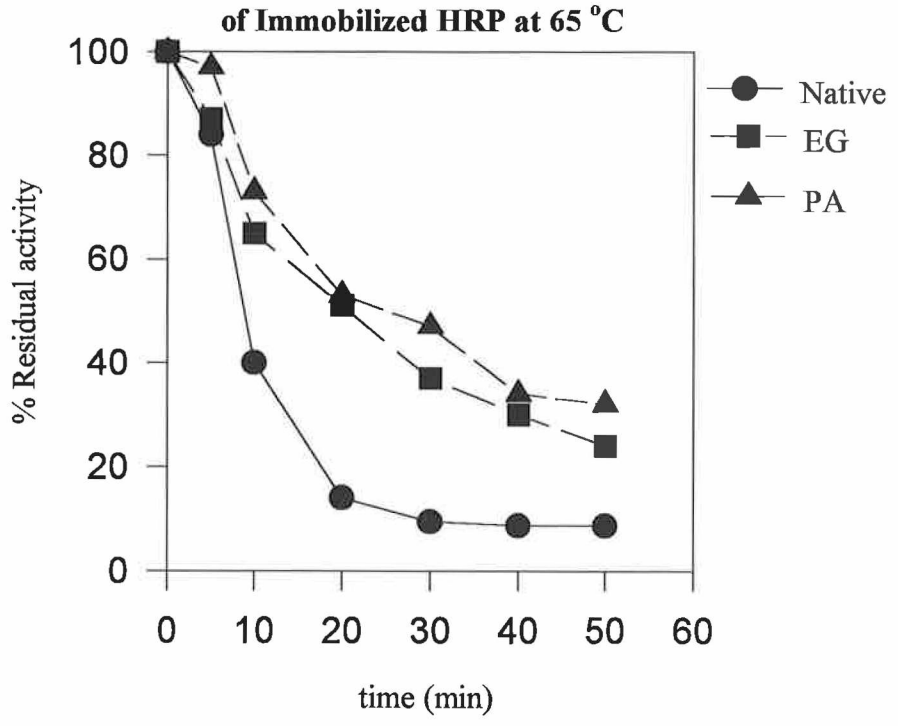


Fig 4.15 **Thermoinactivation of Native and Modified forms of Immobilized HRP at 65 °C**



CHAPTER 5

UNFOLDING HRP

5.0 Unfolding HRP

5.1 Introduction

Horseradish peroxidase is a metalloprotein which contains two moles of calcium per mole of enzyme and it has been found that this calcium is essential in maintaining the structural stability of the protein. The calcium can be removed by incubation with guanidine hydrochloride and EDTA (Haschke and Friedhoff 1978; Pappa and Cass, 1993). Haschke and Friedhoff (1978) reported that this results in reduced thermostability and activity. Urea is another frequently used denaturant. However, denaturation of HRP is usually performed using guanidine hydrochloride, because even in the presence of high concentrations of urea (8M) HRP does not unfold completely. The haem component is also necessary for HRP activity, however, Pappa and Cass (1993) noted that HRP could adopt its secondary structure even in the absence of haem. Therefore, the presence of haem is not necessary for some folding to occur. It has been suggested that haem remains bound to HRP at denaturant (GdCl) concentrations of 1-5M, although the backbone is partially unfolded. At higher concentrations, however, the haem is totally lost. The nature of this second step is not fully understood. Pappa and Cass, (1993) determined the changes in the haem environment during denaturation by examination of the haem Soret absorbance. The effect of increasing concentrations of guanidine hydrochloride was determined on the activities of native HRP and two derivatives (EG-NHS and PA-HRP). The degree of denaturation depends on the enzyme, as co-factors and disulphide-bridges may strongly influence the stability so that extreme conditions may be required for unfolding. Thus HRP and its derivatives were also exposed to reducing agents.

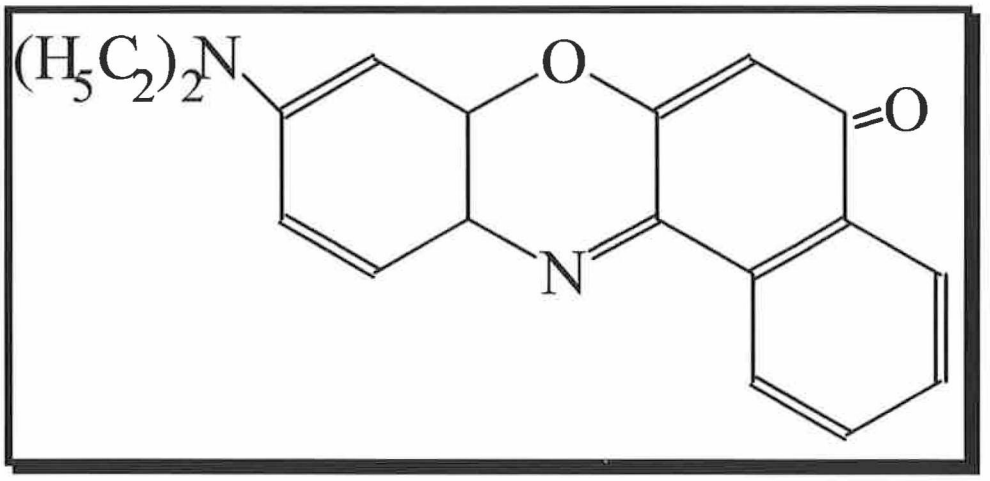
Nonpolar groups are normally found buried in the interior of the molecule, but can become exposed as a result of unfolding, due to the effect of denaturants or as a result of high temperatures. These hydrophobic sites are important in understanding the stability of a protein. A method, sensitive to change in polarity of the protein, was required to confirm that unfolding was occurring. Sackett and Wolf (1987) developed a simple method of detecting changes in the structural integrity of a protein involving the

use of fluorescent hydrophobic probes. These probes are based on the stronger affinity of the probe for the unfolded state, due to the absence of rigid packing of hydrophobic clusters in the unfolded protein). A folded protein has buried hydrophobic clusters and less affinity for a probe molecule.

Nile Red is an uncharged phenoxazone dye (Fig. 5.1), the fluorescence of which is strongly influenced by the polarity of its environment. Nile Red was found to be a useful probe of hydrophobic sites on most proteins. Unlike other probes, it is not limited by ultraviolet or near-UV maxima. Therefore, interferences from biological molecules, the presence of charged groups on the probe, or problems of photo- or oxidative decomposition are avoided. The significant Stokes shifts observed in emission wavelength upon binding to a hydrophobic cluster allow the detection of small changes in protein structure.

Ugarova et al. (1981) observed that conditions altering the protein conformation in HRP also altered the fluorescence of tryptophan in HRP. The fluorescence can some times be quenched by the haem. Ohlsson et al. (1986) calculated the distance of the tryptophan from the haem to be 1.8nm, while Pappa and Cass (1993) predicted that the distance was in fact closer to 1.3nm from the nearest haem edge. Pappa and Cass (1993) used the degree of tryptophan fluorescence to determine the extent of denaturation due to guanidine hydrochloride. However, tryptophan fluorescence is used here as a measure of the extent of thermoinactivation. The principle is similar: as the protein backbone unfolds due to thermal inactivation, the single tryptophan residue, which is buried deep within a hydrophobic region becomes more exposed and the fluorescence intensity increases. This increased fluorescence intensity can be monitored using a fluorimeter.

Fig. 5.1 Chemical Structure of Nile Red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one).



5.2 Results

5.2.1 Effects of Exposure to Urea

The degree of unfolding, indicated by the % activity remaining after the incubation period, was determined for urea as described in Section 2.16.1. The stock solution of urea was prepared as described previously. Native HRP did not completely unfold even in the presence of 8M urea. After 1h at room temperature, in 8M urea, 94% activity remained, even in the presence of 1mM EDTA. The inclusion of 1mM EDTA together with a 2h incubation period had a minimal effect on HRP, reducing the activity to 75% .

Fig. 5.2 **Effects of EDTA on native HRP and two derivatives in the presence of 4.5 M GnCl for 1 hour**

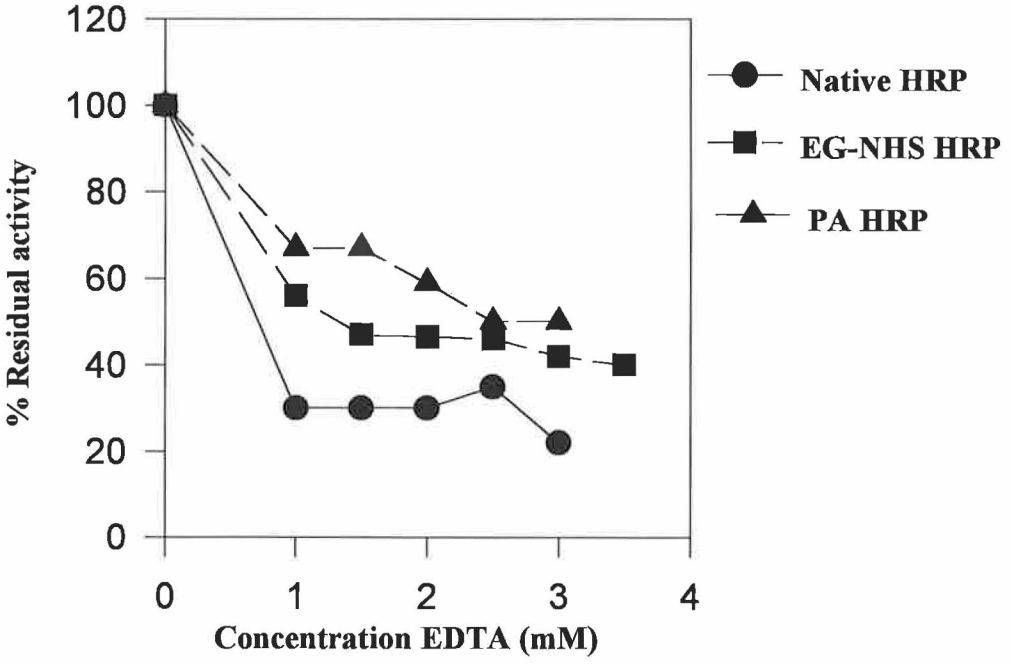
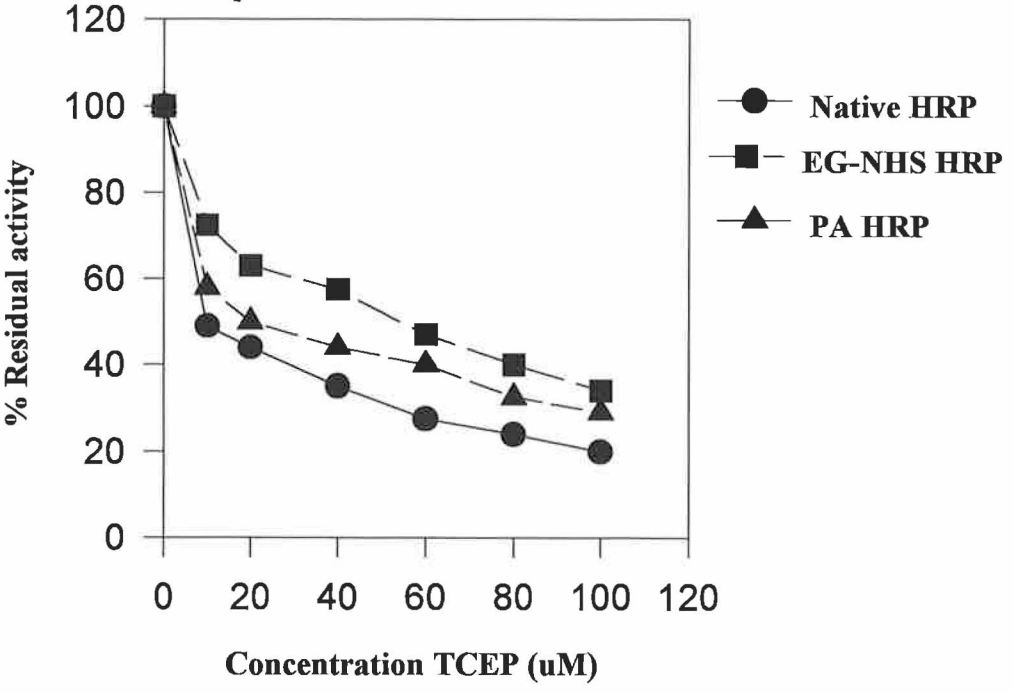


Fig. 5.3 **Effect of TCEP on native HRP and two derivates in the presence of 4.5 M GdnCl for 1 hour**



5.2.2 Effects of Exposure to Guanidine Hydrochloride (GnCl)

Denaturation of HRP and its two derivatives EG-NHS and PA HRP was performed as described in Section 2.16.1. From denaturation curves for HRP incubated at room temperature for one hour it was noted that between 0-2M GnCl the enzyme activity is enhanced. The optimum range for unfolding lay between 4-6M GdnCl where a significant drop in activity was observed. There is a marked increase in stability towards GnCl for the modified forms compared with the native while C_{50}, (the concentration at which 50% of the activity is lost) = 4.5M for the native, C_{50} = 6M for both the EG-NHS and the PA. (Table 5.1).

5.2.3 Stability Towards Chelating and Reducing Agents

4.5M GnCl was chosen as the denaturant concentration giving approximately 50% unfolding at room temperature for native HRP. The procedure was carried out as described in Section 2.16.3. Both HRP derivatives demonstrated enhanced stability towards both EDTA and TCEP (Fig. 5.2 and 5.3). There was almost 3-fold greater stability for PA HRP exposed to EDTA compared with native HRP. (Table 5.1). On exposure of the samples to EDTA there was an initial sharp decrease up to 1mM concentration. Increasing the EDTA concentration further only had a slight effect on the activities, whereas the degree of unfolding upon exposure to TCEP was more gradual and followed a single exponential decay curve.

Table 5.1 Comparison of C_{50} Values for Native, EG-NHS and PA HRP with Denaturants/Reducing/Chelating Agents

Reagent	HRP C_{50}	EG-NHS C_{50}	PA HRP C_{50}
GnCl (M)	4	6.3	5.9
EDTA (mM)*	1.1	2	3
TCEP (μM)*	28	65	53

* in the presence of 4.5M GnCl

5.2.4 Effects of Temperature on Nile Red Fluorescence for Native, EG-NHS and PA HRP

The reaction with Nile Red was performed as described in Section 2.17.2. Heat treatment resulted in a progressive change in the HRP, in the form of a blue shift from 640nm to 620nm. The increase in temperature also resulted in a significant increase in the fluorescence intensity (Figs.5.4-5.6). The highest temperature (up to 85 °C for these experiments) gave the largest increase in emission readings and the greatest shift towards 620nm. When the maximum fluorescence intensities for the two derivatives were compared it was noted that the EG-NHS HRP gave the lowest yield in fluorescence, and the fluorescence for the PA HRP was greater than for the native. The fluorescence intensities at 35 min were 12, 7.5 and 12.5 for the native, EG-NHS and PA HRP respectively (Fig. 5.7). The native HRP was also incubated at a range of temperatures for a period of 10 min and the Nile Red fluorescence monitored. As can be seen in Fig. 5.8, fluorescence intensity increased with temperature and the characteristic blue shift was also evident. Buffer was also incubated at room temperature and at 85 °C but there was no significant increase in fluorescence. Therefore the increase in fluorescence is indeed due to a structural change in the HRP

Fig. 5.4 **Fluorescence of Nile Red in the presence of native HRP at 85 °C at different time intervals**

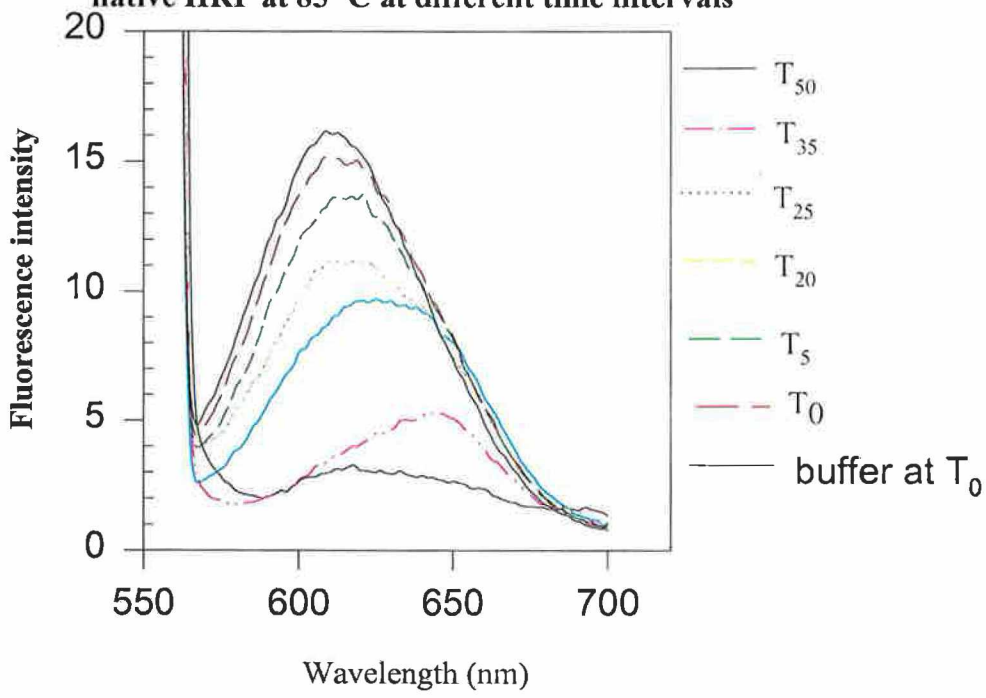


Fig. 5.5 **Fluorescence of Nile Red in the presence of EG HRP at 85 °C at different time intervals**

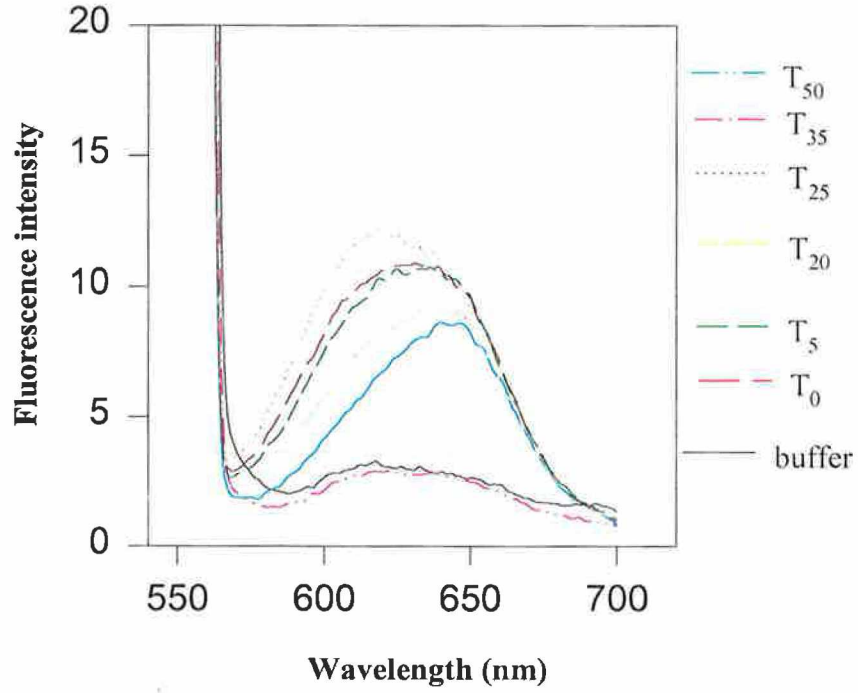


Fig. 5.6 **Fluorescence of Nile Red in the presence of PA HRP at 85 °C at different time intervals.**

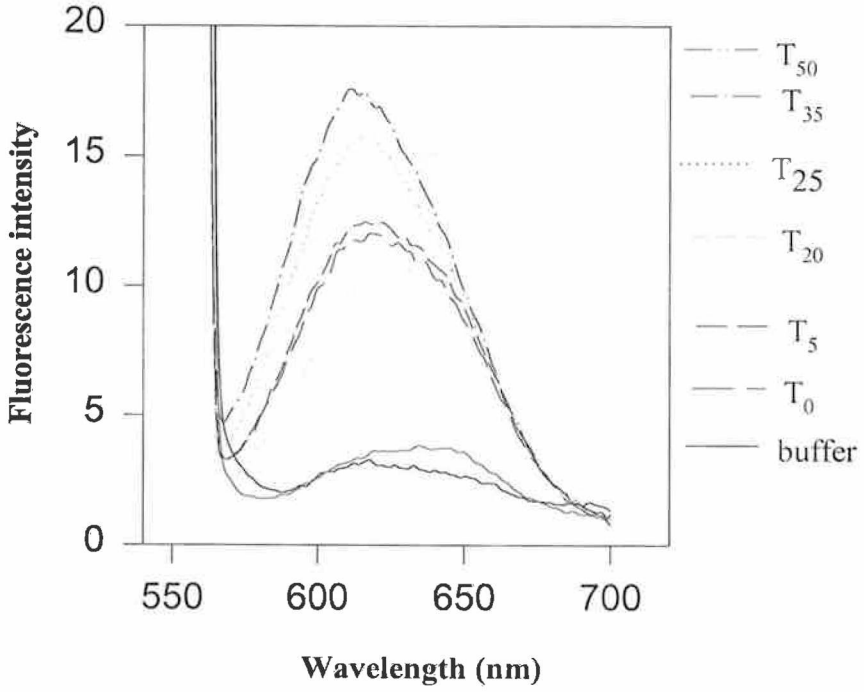


Fig. 5.7 **Maximum fluorescence vs time at 85 °C**

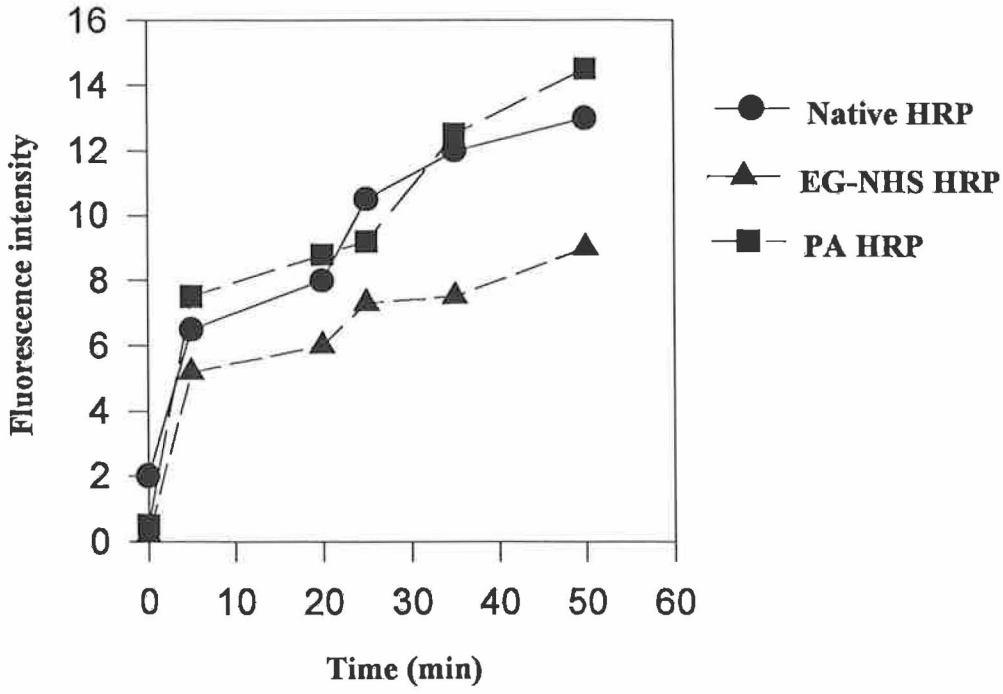
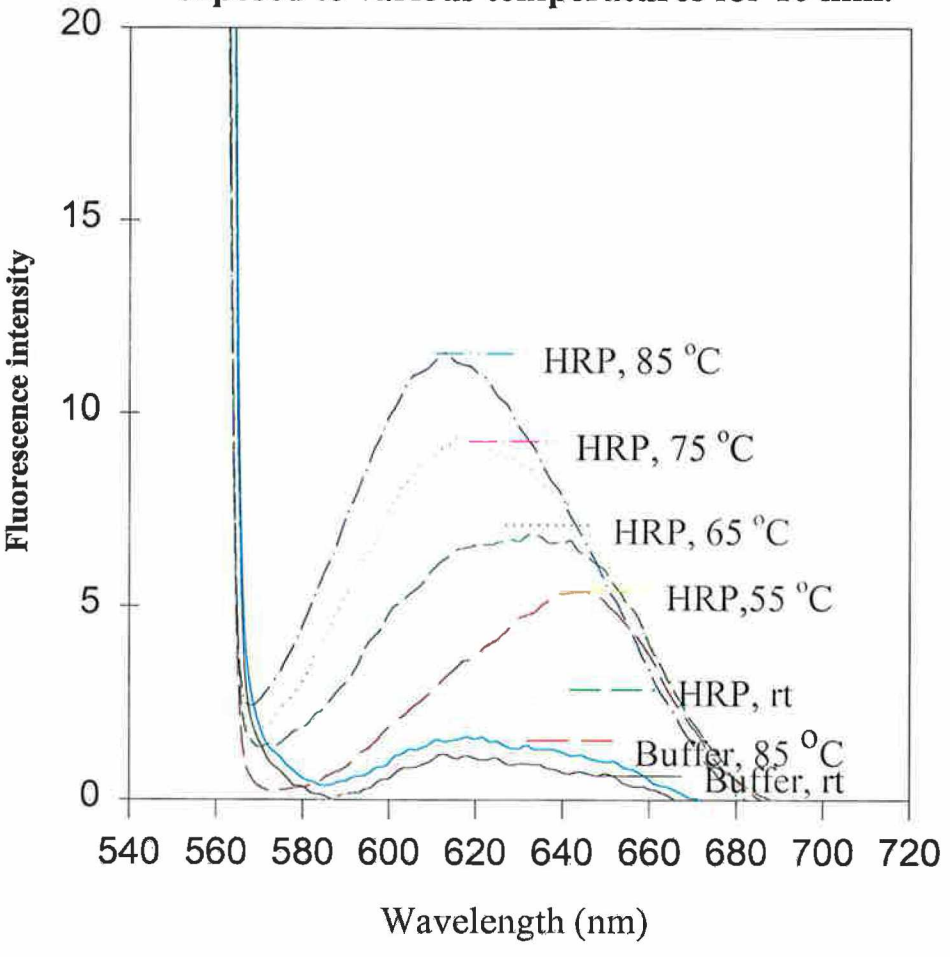


Fig. 5.8 **Fluorescence of Nile Red in the presence of native HRP exposed to various temperatures for 10 min.**



5.2.5 Tryptophan Fluorescence Due to Heat-Induced Unfolding

5.2.5.1 Preparation of an L-Tryptophan Standard Curve

The fluorescence intensity due to tryptophan was determined as described in Section 2.17.1. Initially a standard curve of free L-tryptophan was prepared in the range 0-10μM in distilled water. The fluorescence intensity was measured after incubation at room temperature and at 65 °C for 90min (Fig. 5.9). There was a very small difference in fluorescence intensity between the two temperatures, with only a slight decrease in intensity at 65 °C. A second standard curve was then prepared in the same concentration range; however, this time the standards were prepared in 3.5 μM native HRP. Fluorescence was measured after 90 min incubation at room temperature. The slope of the line in this instance was 21.5 compared with a slope of 53.5 for the previous standard curve prepared in buffer alone. Therefore, there was a quenching factor of 2.45 in the presence of 3.5μM HRP. (Fig. 5.10). The latter curve was taken as the working standard curve.

5.2.5.2 Effect of Heating on Fluorescence of Native, EG and PA HRP

The procedure was carried out as described in Section 2.17.1. The fluorescence intensity for native HRP was much greater than for the modified forms (Fig. 5.11). The concentration of tryptophan measured after 90 min was estimated to be 4.4μM, 2.4μM and 3μM for the native, EG and PA HRP respectively as determined from the standard curve. This is within the range that would be expected for the native HRP as the concentration used was 3.5μM. There is less fluorescence for the modified forms as they have been prevented from completely unfolding by the respective modifications. They have in effect been protected from the denaturing effect of the excessive heat.

The fluorescence intensity was compared with the % relative activity over the same time period and at the same temperature. It can be clearly observed that as the fluorescence increases, the relative activity decreases. The native displays a large increase in fluorescence over the incubation period with an initial sharp increase followed by a

Fig. 5.9 **Tryptophan standard curve at room temperature and at 65 °C for 90 min**

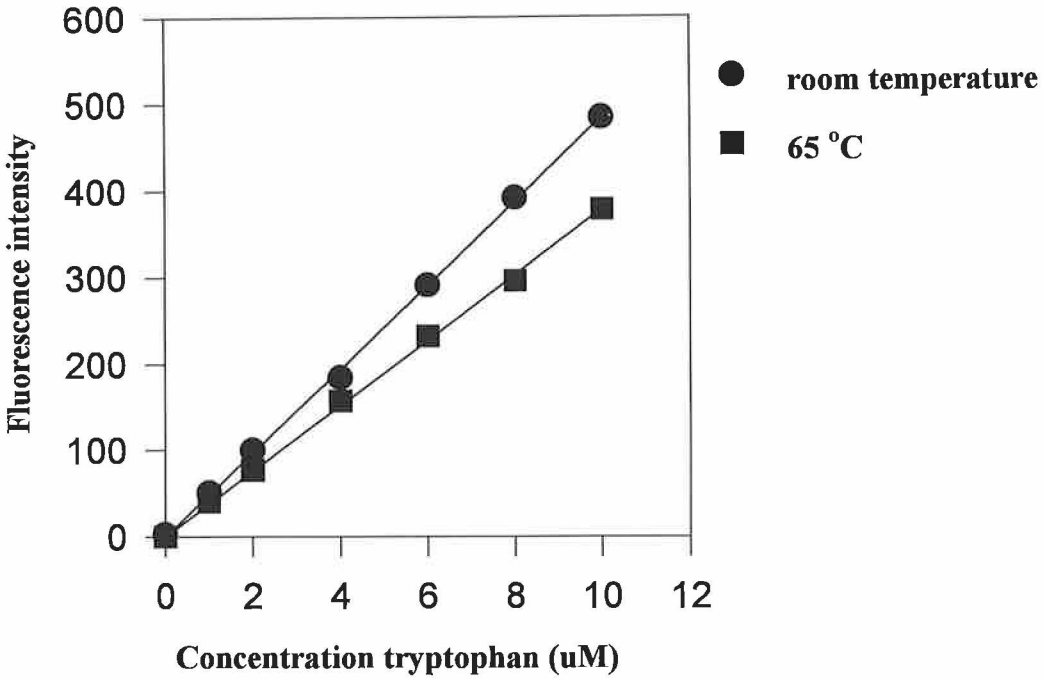


Fig. 5.10 **Tryptophan standard curve at room temperature** in the presence of 3.5uM HRP

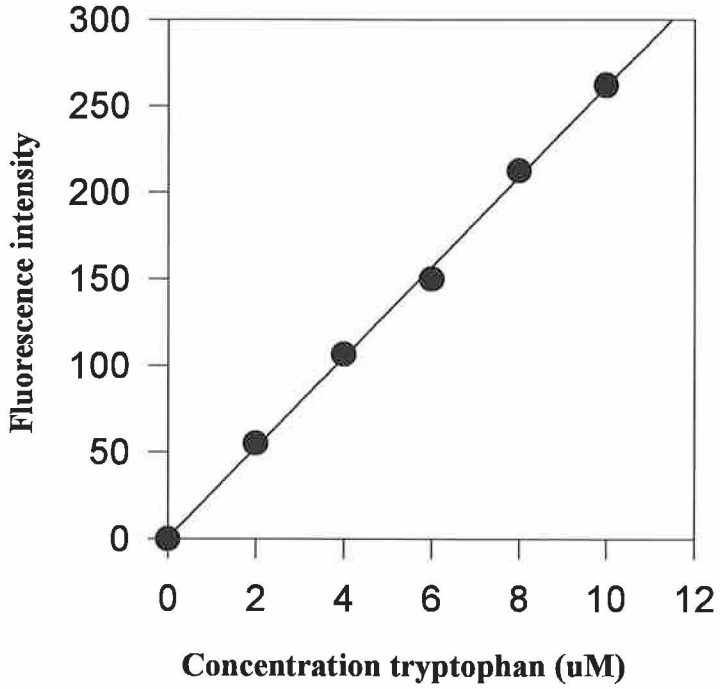
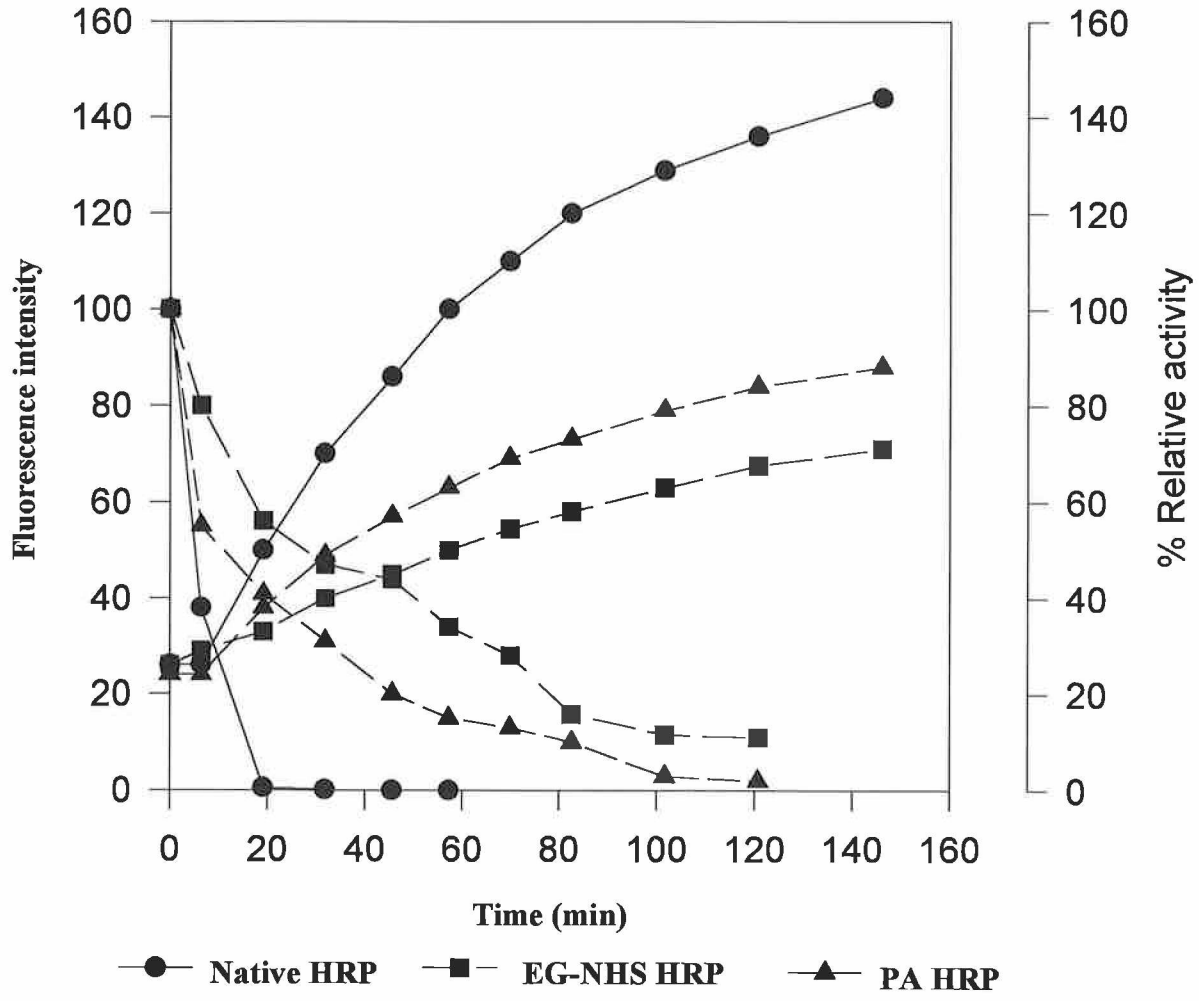


Fig. 5.11 **Fluorescence vs relative activity at 65 ^{0}C over time**



more gradual increase. this corresponds to the activity curve where after 20 minutes at 65 °C the relative activity has diminished to practically zero. For both of the modified forms, the fluorescence increase is less dramatic, gradually increasing to approximately 70 and 88 fluorescence units for the EG and the PA respectively. In the case of the EG HRP this is less than half the value for the native HRP. Comparing these results to the % relative activity for the modified forms, it is noted that the activity curves decay slowly and even after 120 min have not reached zero.

5.3 Digestion of Holo/Apoenzymes

5.3.1 Preparation of Apo-Enzyme

The haem component of the HRP was removed as described in Section 2.12. The yield of apo-enzyme was high, in excess of 80% as determined by the BCA assay (Section 2.3.1.1).

5.3.2 Digestion of HRP

The holo (intact) and Apo-enzymes were incubated with Cyanogen bromide, Endo-lys C or trypsin as outlined in Sections 2.13, 2.14 and 2.15.

5.3.3 SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out as described in Section 2.2. The stained gels showed bands in a similar position for control (native) , EG-NHS and PA HRP. The enzymes did not show up as a single band at 44,000Da; rather there were multiple bands present indicating that the enzymes were not pure. As Fig. 5.12a shows, for the native apo-enzyme a number of extra bands were noted indicating the native HRP had been fragmented by exposure to the cyanogen bromide. Cyanogen bromide cleavage for both of the modified forms resulted in the absence of any bands. For the trypsin digestion, there was no difference between the trypsin samples and

bands. For the trypsin digestion, there was no difference between the trypsin samples and the control samples. A band did appear on the trypsin samples which was later determined to be soybean trypsin inhibitor. The result for the endo-Lys C digestion was similar to that of the cyanogen bromide; however, there was a band at less than 14,000Da present for the endo-Lys C that was not apparent with the cyanogen bromide. (Fig.5.12b).

Fig. 5.12a SDS-PAGE of Native, EG and PA Apo -HRP (CNBr)

Modified and native HRP (5μg per well in all cases) failed to migrate as a single band for the control samples. The native/CnBr appears as a number of bands. The molecular weight markers on the left were (from top) bovine serum albumin (66,000Da), egg albumin (45,000Da), glyceraldehyde-3-phosphate dehydrogenase (36,000Da), carbonic anhydrase (29,000Da),bovine trypsinogen (24,000Da) and soybean trypsin inhibitor (20,100Da). Some extra molecular weight markers were included in lane 2 these were insulin A and B chains(2,531Da and 3,495Da), lysozyme (14,200Da) and cytochrome C (13,000Da).

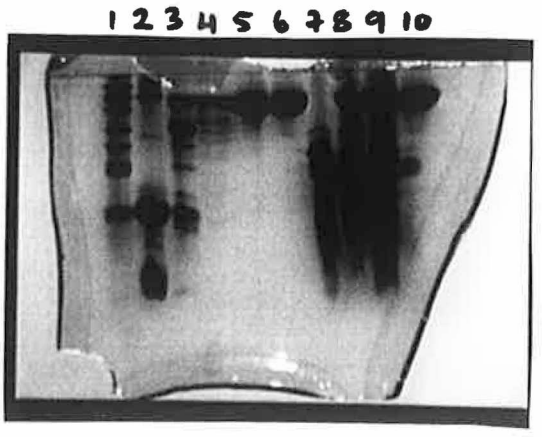


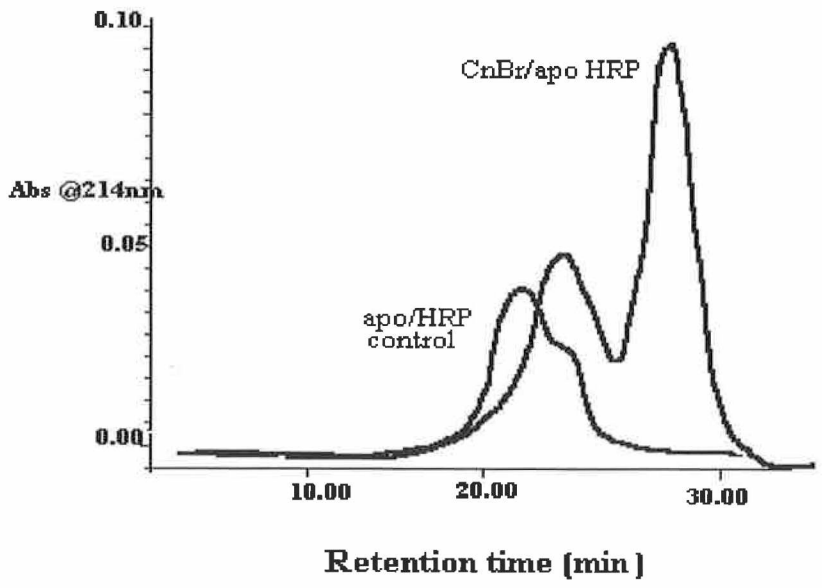
Fig. 12b SDS-PAGE for Holo and Apo-Native HRP (Endo.lys C)

The apo-native HRP incubate with endo.lys C (5μg per well) migrated as a number of bands with one low molecular weight band. The control HRP and the holo HRP did not appear as single bands. The molecular weight markers used were the same as the cyanogen bromide gels (Fig.5.12a).

5.4 HPLC Analysis of Cyanogen Bromide Fragments

The HPLC analysis of the cyanogen bromide fragments for the apo native EG-NHS and PA HRP was carried out as described in Section 2.2.22.1. The profile for the apo-native control and following incubation with cyanogen bromide is shown in Fig. 5.14. As can be seen in the profile, there is a shoulder present on the peak for native control (no cyanogen bromide), indicating that the sample is not pure; this is in agreement with the SDS-PAGE results. However, only two peaks were detected for the cyanogen bromide fragment and these were a higher intensity than the control. The EG-NHS and PA HRP gave similar results (profiles not shown). It was difficult to draw any conclusions from the data obtained in the HPLC analysis.

Fig. 5.14 Schematic of Cleavage of Native Apo-HRP by Cyanogen Bromide



Note: HPLC analyses were carried out as described in Section 2.22.1

CHAPTER 6

RECOMBINANT HORSERADISH PEROXIDASE

6.0 Recombinant Horseradish Peroxidase

6.1 Introduction

Over the past twenty years, manipulation of DNA *in vitro* has developed from the transfer of genetic information between prokaryotic organisms to a technology that facilitates efficient and controlled production of proteins in foreign hosts (Cohen et al, 1973). One of the main features of this development is the ability to express proteins in prokaryotes such as *E. coli*. therefore, gene cloning and expression in *E. coli* can provide an abundant source of valuable proteins and also affords the opportunity to perform mutations which could be of benefit to the protein or help elucidate the structure and mechanisms of action.

The mode of gene expression affects the location of proteins produced. They can be (a) located in the cytoplasm or (b) secreted through the cell membrane. An approach which locates proteins in the cytoplasm (direct expression) uses bacterial promoters and terminators in the transcription of the foreign gene alone (Marston, 1986). Directly expressed proteins often posses an unnatural N-terminal methionine residue. This is because, although *E. coli* possesses an enzyme which catalyses the efficient removal of the methionine residue, it does not always work on the recombinant protein. In general recombinant polypeptides accumulate to higher levels of total cell protein when expressed intracellularly than when excreted. Many of the polypeptide products located in the cytoplasm are insoluble and aggregated. Expression, isolation, purification and refolding of the recombinant enzymes are discussed in more detail in Chapter 1.

6.2 Results

6.2.1 Effect of Temperature on HRP C*

The thermostability of HRP C* was compared with that of the commercial plant HRP as described in Section 2.8.2. HRP C * appeared more thermostable under the conditions employed than the commercial plant enzyme (Table 6.1 and Fig 6.1). Thermoinactivation of the various HRPs at 65 °C fitted a first-order exponential decay.

6.2.2 Chemical modification of HRP C*

The recombinant HRP was modified with ethylene glycol-bis-N-hydroxysuccinimide ester as outlined in Section 2.4.1. The modification of HRP C* with EG-NHS was accompanied by enhanced activity (136.5 ±12.02%).

6.2.3 Effects of Temperature on Modified HRP C*

Thermostability of modified HRP C* was compared with native HRP C* and native plant HRP as described in Section 2.8.2. Chemical modification increased the 65^0 C half-life of HRP C* 5-fold (Table 6.1). HRP C* maintained in excess of 70% residual activity at 65 °C for up to 50 min. (Fig. 6.1). The residual activity was compared with that of the native over the temperature range 20-80 °C as described in Section 2.8.1. The modified recombinant and recombinant HRPs retained as much as 38% and 15% activity respectively at 70 °C. The native plant HRP had lost all activity at this temperature, (Fig. 6.2)

6.2.3 Stability of EG-NHS-HRP C* /HRP C* in Organic Solvents

Organotolerances of EG-HRP C* and HRP C* and native HRP were determined as described in Section 2.9 in DMF and 1,4-dioxane. No enhanced tolerance of DMF was noted for the recombinant and its EG derivative. However, in 1,4-dioxane there was a notable difference. The activity was enhanced by up to 20% and almost 100% activity was maintained up to 40% solvent. The C_{50} values increased from 52 % for plant HRP to 70% for the modified recombinant (Fig.6.3).

TABLE 6.1 Thermoinactivation of Horseradish Peroxidase Forms at 65 °C.

HRP Type	k, (min^{-1})	$t_{1/2}$, (min)	Stabilisation (fold)
Plant	0.147 +/- 0.01	5	----
	0.175 +/- 0.007	4	----
HRP C*	0.032 +/- 0.003	22	1
	0.033 +/-0.007	21	1
EG-HRP C*	0.006 +/- 0.002	114	5
	0.0064 +/-0.0003	108	5

HRP TYPES: Plant, commercial horseradish peroxidase (Boehringer Mannheim);
HRP C* = recombinant horseradish peroxidase (Smith et al., 1990);
EG-HRP C* = recombinant HRP modified with EG-NHS.

Fig. 6.1 Thermostability studies on native HRP and HRP C* derivatives at 65 °C

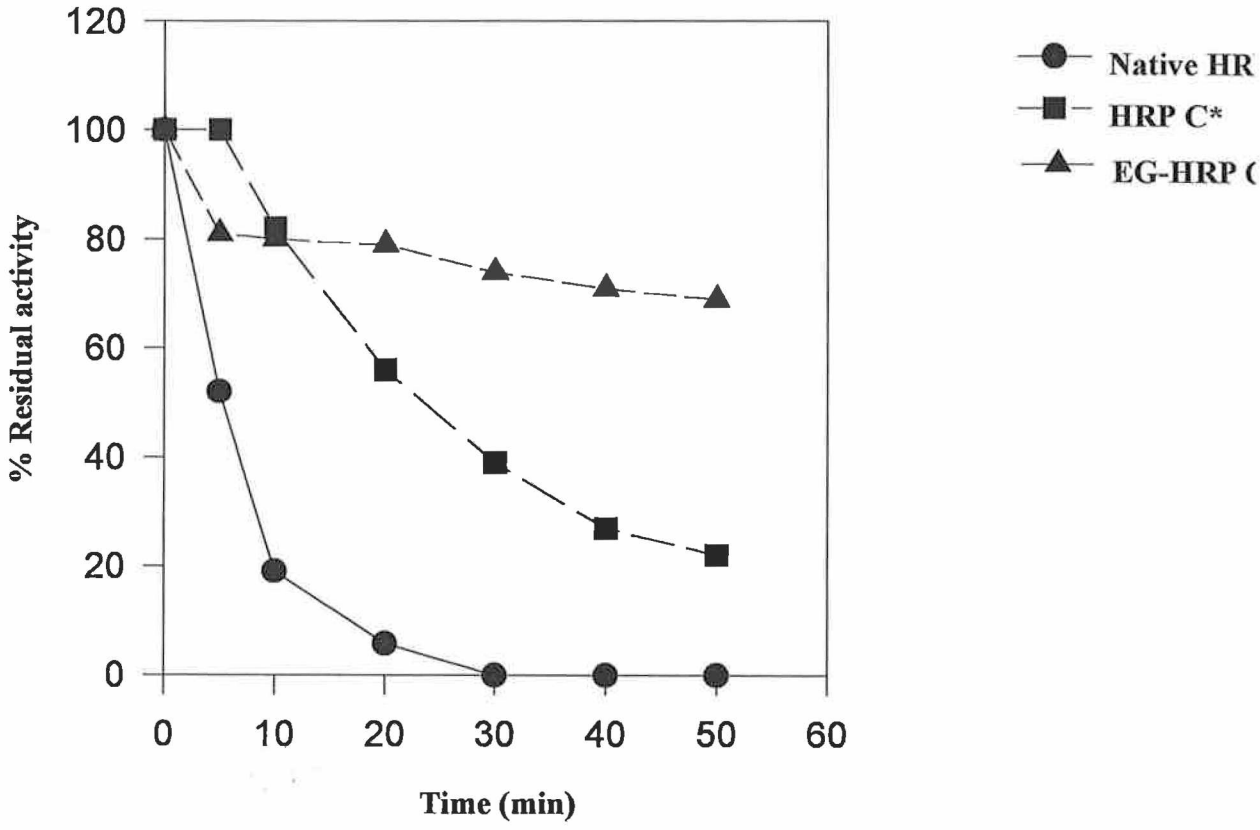


Fig. 6.2 **Temperature profile for native plant HRP, HRP C* and EG-HRP C***

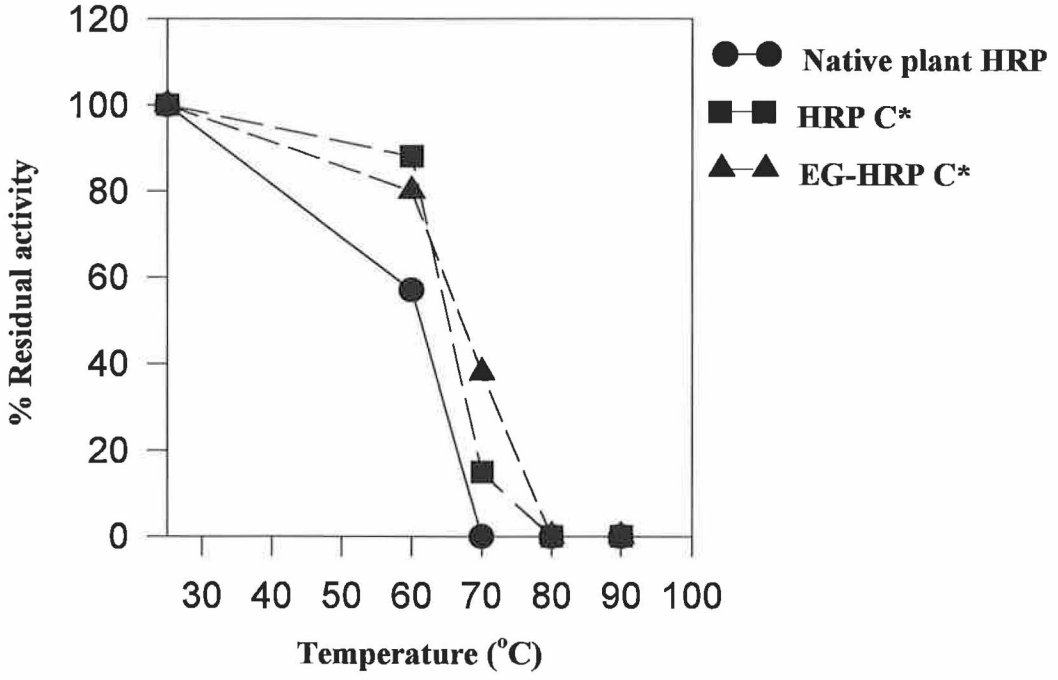
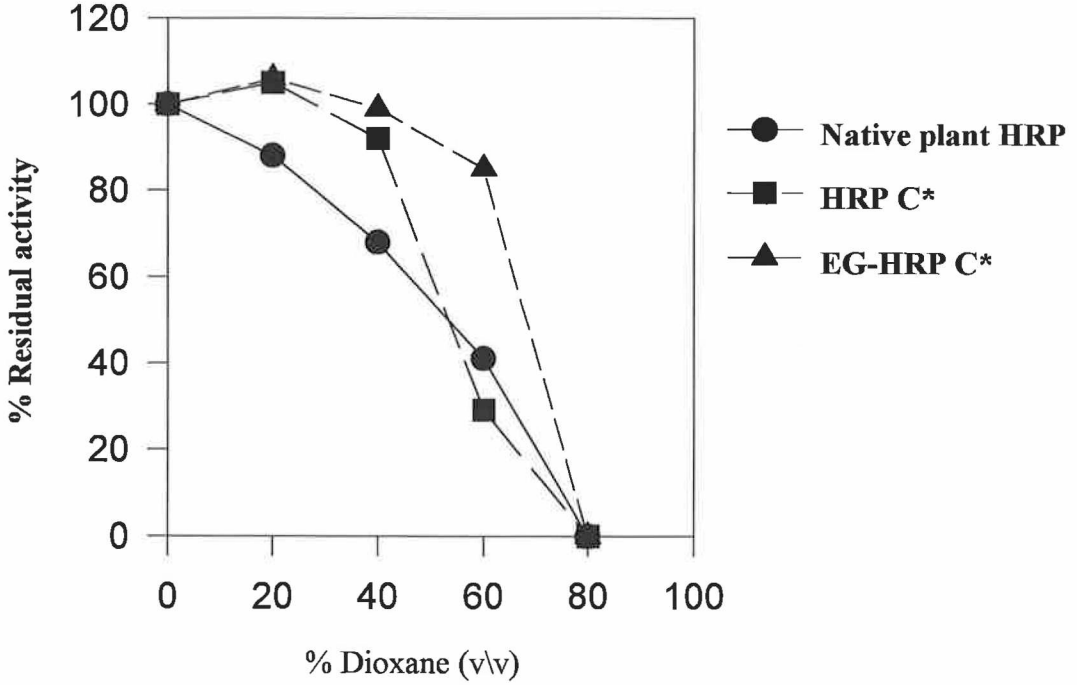


Fig. 6.3 **Effect of Dioxane on native plant HRP, HRP C* and EG-HRP C***



CHAPTER 7

KINETIC STUDIES ON NATIVE AND MODIFIED HRP

7.0 Kinetic Studies on Native and Modified HRP

7.1 A Study of Benzhydroxamic Acid Binding to Native, EG-NHS and PA HRP

7.1.1 An introduction to binding studies

All proteins carry out their biological function through the formation of complexes with other molecules. Proteins may form complexes with cellular structures, other macromolecules (including other proteins), substrates, inhibitors, or other small molecules. For the sake of general discussion, anything forming a complex with a protein is often simply referred to as a 'ligand' by the protein chemist. Proteins may have a single binding site or many binding sites. Multiple binding sites may be identical or differ and may interact in a co-operative (as is the case for allosteric enzymes) or non-co-operative manner. In order to understand the mechanisms of action of enzymes and of regulatory systems, a biochemist will often need to know the number of binding sites on a protein, and the strength of binding. Simple algebraic analysis of ligand binding to protein has been carried out and has been summarised and outlined simply by Sanders (1994).

7.2 Results

7.2.1 Spectral Changes on the Addition of BHA to HRP and its Derivatives

Benzhydroxamic acid (BHA; Fig. 7.1) is a known inhibitor of HRP and its mode of binding to the enzyme has been extensively studied. Its binding to HRP is characterised by an intensification of, and a shift from, 402 nm to 407 nm of the Soret peak The Soret peak also narrows with the loss of a shoulder at 381nm which is present in the absorbance spectrum of the resting enzyme (Sanders,1994). These changes are typical of a change from a pentacoordinate high spin-haem iron to a hexacoordinate high spin haem iron, as discussed in Section 1.2

In the present work, the changes in the absorbance spectra on the addition of BHA to HRP, EG-NHS HRP and PA HRP in 10 mM phosphate buffer pH 7.0 were almost identical, with the three enzymes displaying the well documented spectral changes associated

with the binding of BHA to HRP. Very little change in the visible absorption spectra of the three enzymes was noted. Below 300 nm an increase in the UV absorption spectra can be seen on the addition of BHA. This is, however, due to the absorbance of BHA itself.

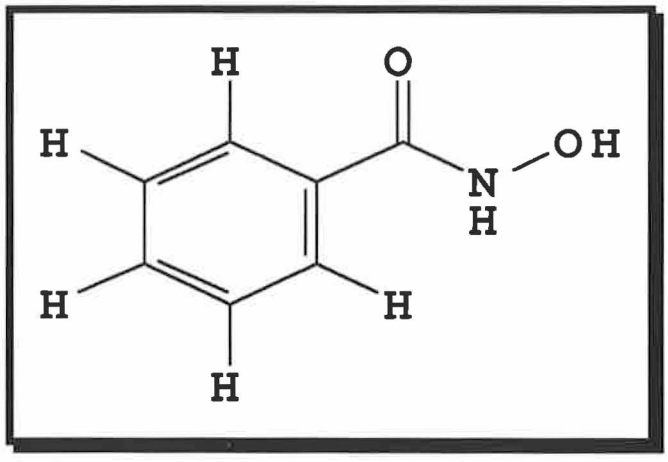


Fig. 7.1. The Structure of Benzhydroxamic Acid (BHA).

7.2.2 Determination of Values of K_d (dissociation constant)

In order to obtain data for the Scatchard analysis of BHA binding to HRP and derivatives (see Fig.7.2 for example of a Scatchard plot for native HRP), solutions of these enzymes in 10 mM phosphate buffer pH 7.0 were titrated over a range of BHA concentrations and observed by difference absorption spectrophotometry of the Soret peak region (Section 2.21) where the absorbance changes are at their greatest (Fig. 7.3 and 7.4).

The maximum change in absorbance in each case occurred at 407 nm in agreement with published data for HRP C. (Smith et al, 1992). The peak heights were then subtracted from each other to determine the difference in absorbance on addition of each aliquot of BHA. (Fig 7.5 + 7.6 illustrate the spectrophotometric profiles obtained)

Fig.7.2

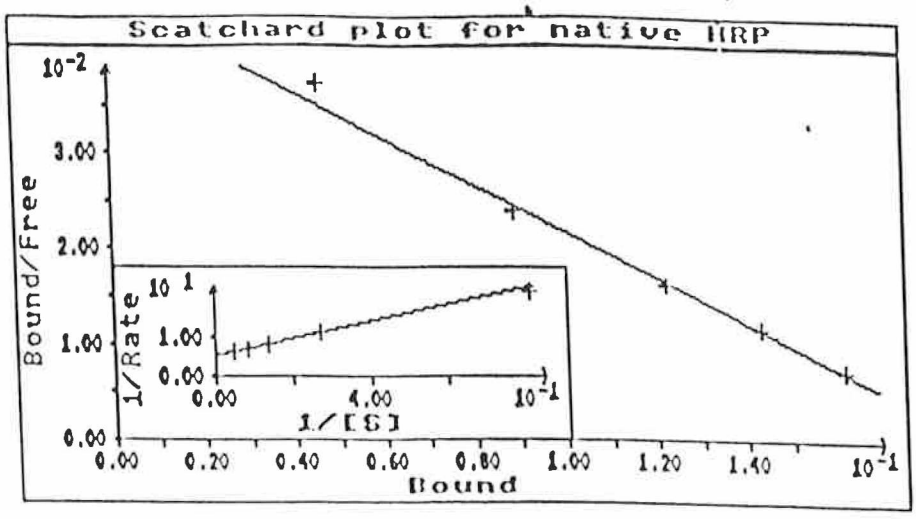
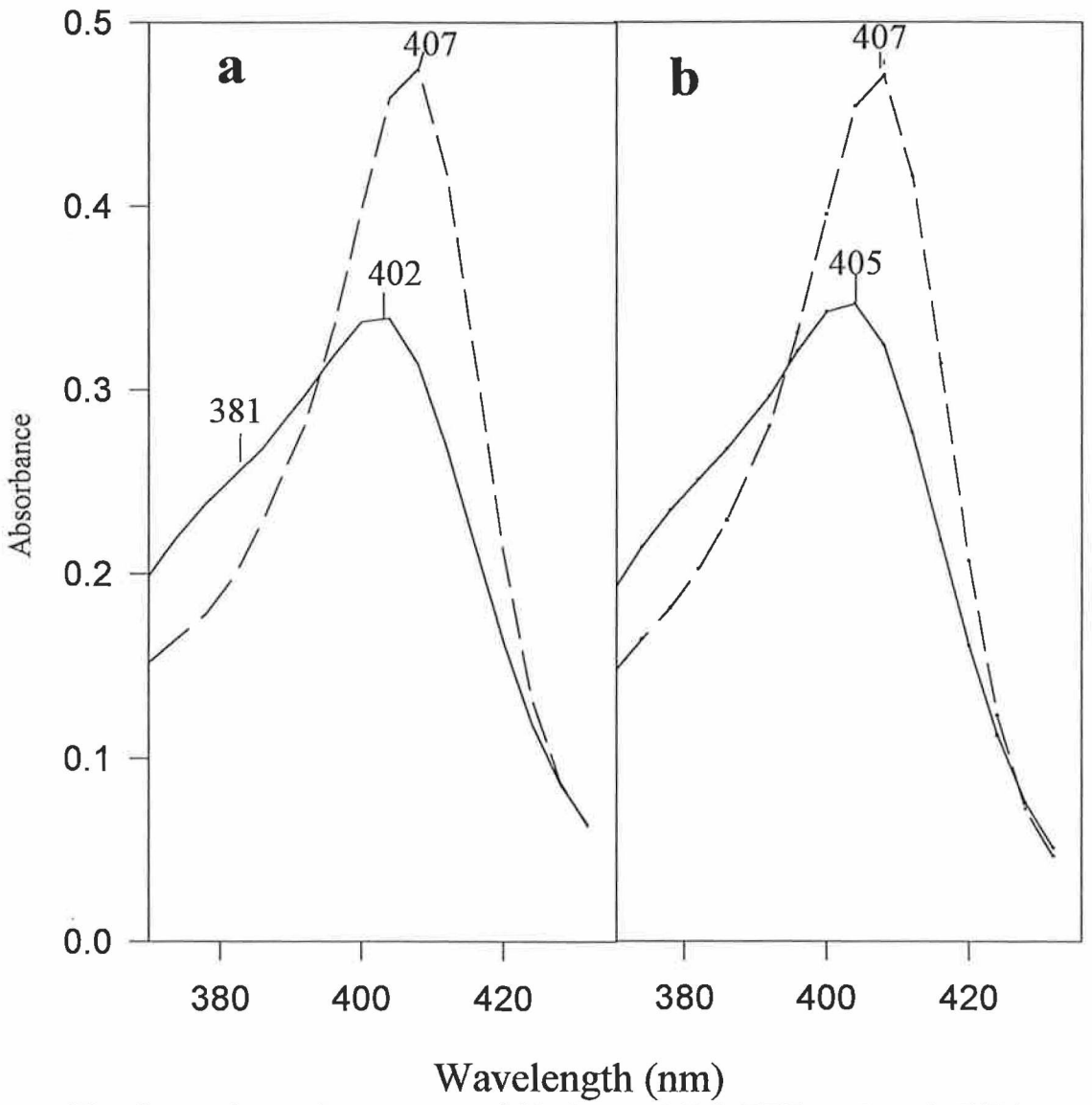


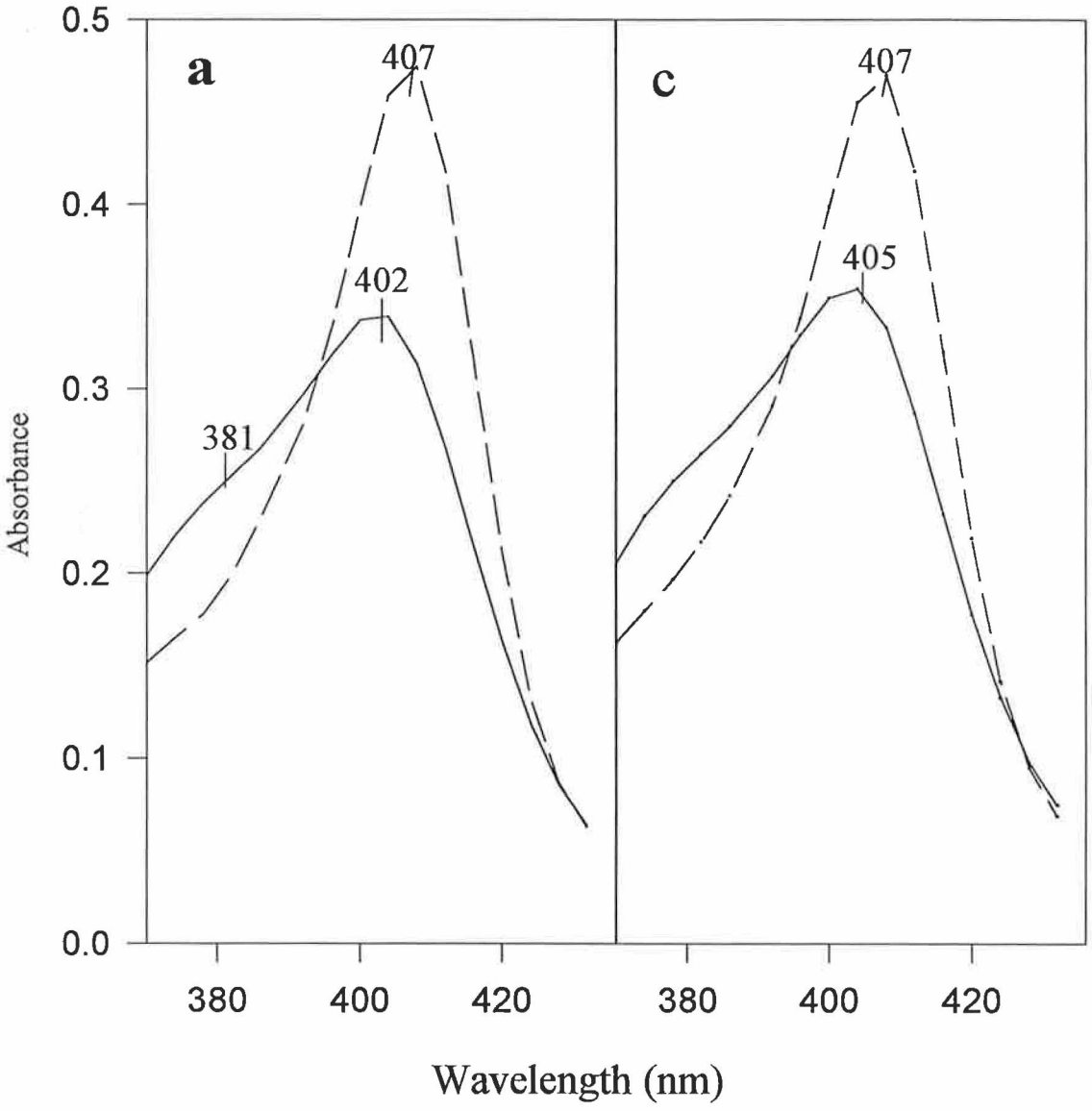
Fig. 7.2. A Scatchard plot of the Binding of BHA to 4.0μM Native HRP. The concentrations of BHA added are 1.24, 3.73, 7.44, 12.34 and 22.0 μM.

Fig. 7.3 **The Soret Absorption Spectra of Native and EG HRP**



The Soret absorption spectra of Native and EG HRP and their BHA complexes in 10mM phosphate buffer, pH 7.0. a) 3.5μM native HRP before (———) and after (-------) the addition of 22 μM BHA. B) 3.5μM PA HRP before (———) and after (-------) the addition of 22 μM BHA.

Fig. 7.4 The Soret Absorption Spectra of Native and PA HRP



The Soret absorption spectra of Native and PA HRP and their BHA complexes in 10mM phosphate buffer, pH 7.0. a) 3.5μM native HRP before (————) and after (-------) the addition of 22 μM BHA. B) 3.5μM PA HRP before (————) and after (-------) the addition of 22 μM BHA.

Fig. 7.5 Spectra Obtained on Titrating native HRP with BHA

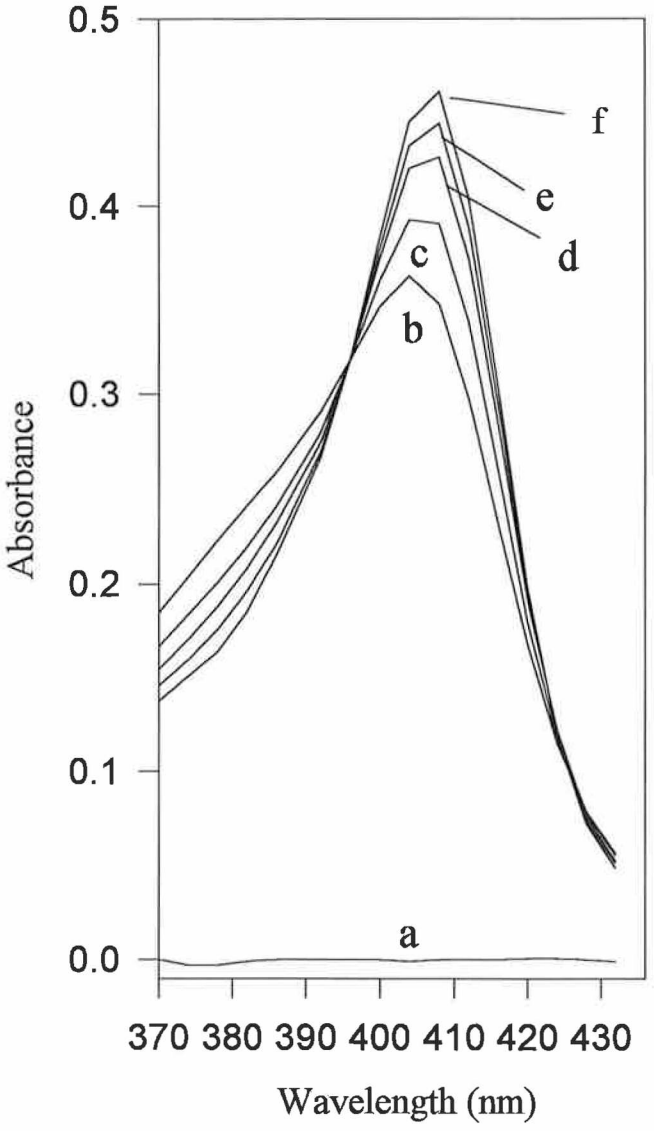


Fig. 7.5 Spectra Obtained on Titrating Native HRP with BHA in 100mM phosphate Buffer, pH 7.0. The reference cell contained 400μL 100mM phosphate buffer, pH 7.0. a) baseline reading. The sample cell contained 400μL of 4μM native HRP and b) 1.24, c) 3.73, d) 7.44, e) 12.34, f) 22.0 μM BHA

Fig. 7.6 **Spectra Obtained on Titrating EG and PA HRP with BHA**

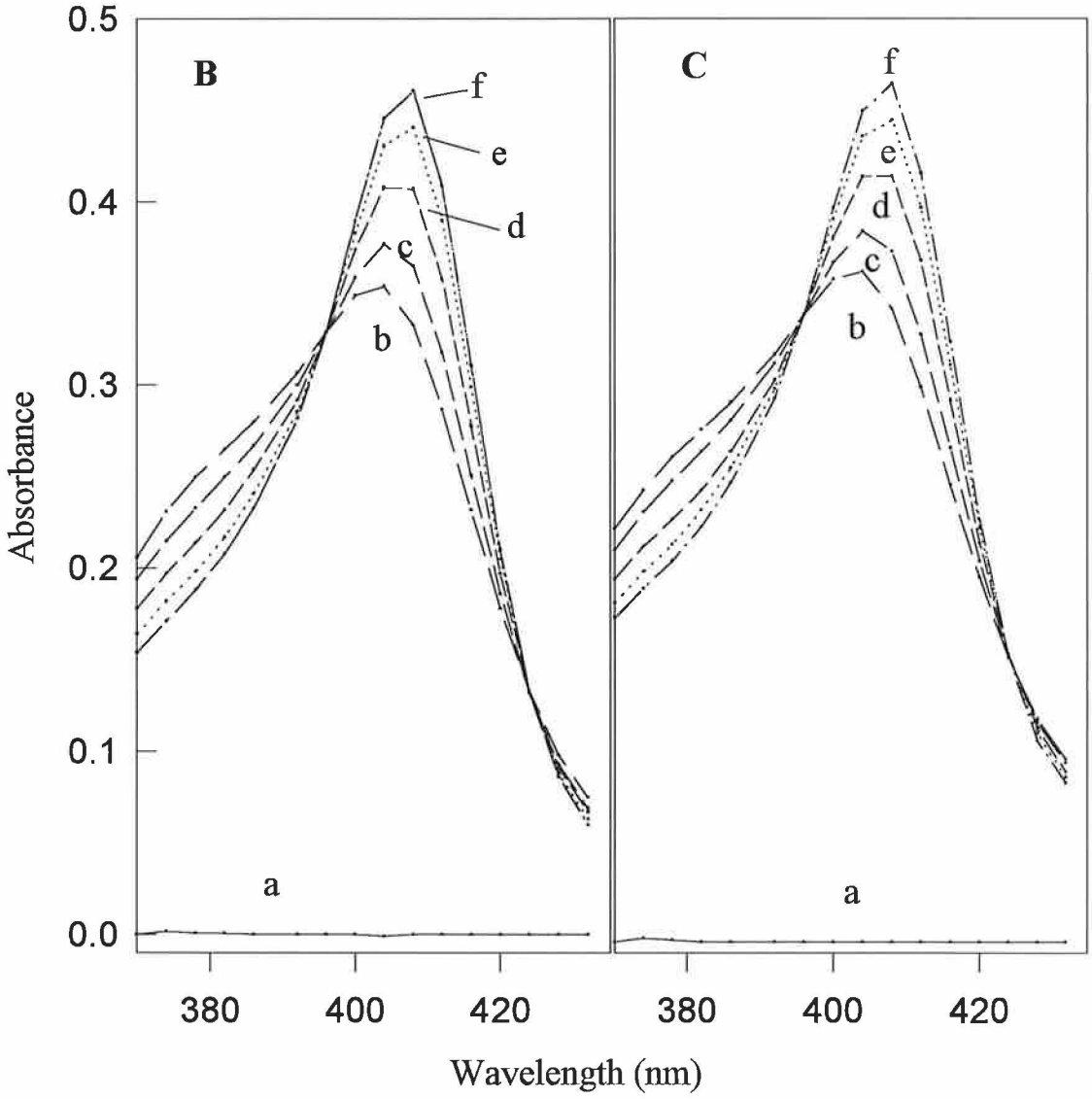


Fig. 7.6 Spectra Obtained on Titrating EG (B) and PA (C) HRP with BHA in 100mM phosphate Buffer, pH 7.0. The reference cell contained 400μL 100mM phosphate buffer, pH 7.0. a) baseline reading. The sample cell contained 400μL of 4μM native HRP and b) 1.24, c) 3.73, d) 7.44, e) 12.34, f) 22.0 μM BHA

The dissociation constant (K_d) was determined from the change in the difference spectrum (treated minus untreated) of the Soret region, resulting from titration of resting enzyme to saturation with the substrate BHA. Results (two determinations) are derived from computer fits of the three variables to the binding equation given in experimental procedures (Section 2..22). The results are shown in Table 7.1.

Table 7.1 Binding of Benzhydroxamic (BHA) Acid to Resting State Peroxidases.

Peroxidase	BHA K_d (dissociation constant, μM)
Native HRP C	2.15 ± 0.36, 1.94 ± 0.48
EG-NHS HRP C	2.41 ± 0.26, 1.8 ± 0.18
PA HRP C	2.54 ± 0.49, 1.92 ± 0.27

The results obtained for the dissociation constant (K_d) correspond well to results published previously for native plant HRP C (Smith et al., 1992; Gilfoyle et al., 1996; and Veitch et al., 1995). These workers obtained values of 2.1, 2.5 and 2.7 μM for K_d^{BHA} for the recombinant HRP.

7.3 Kinetic Studies on Native, EG-NHS and PA HRP

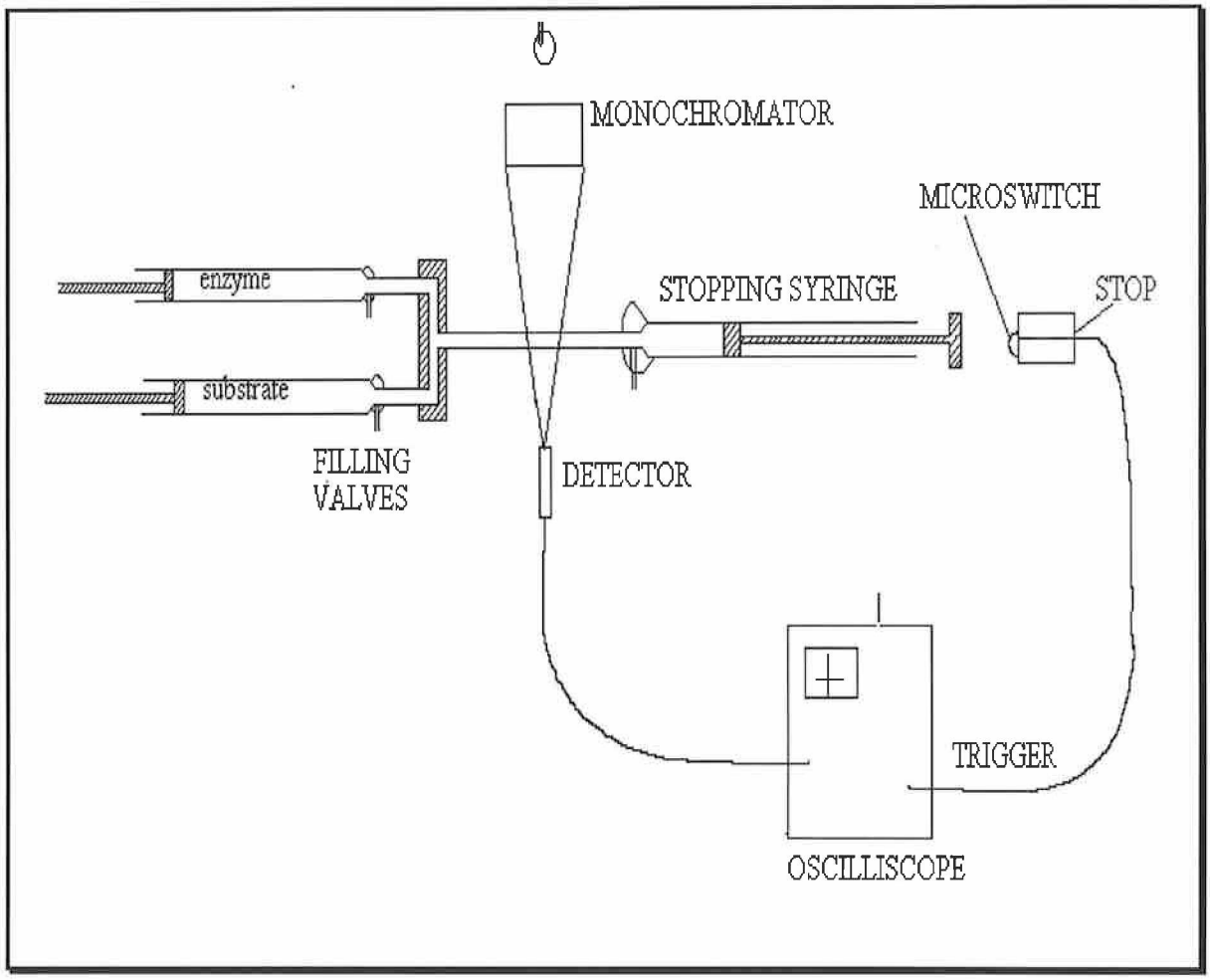
The kinetics of native and both modified forms of HRP were studied to determine the effects of the modifications on the rate constants. The classical catalytic cycle of HRP is shown in Section 1.2.3 Fig.1.2. The reaction of HRP with hydrogen peroxide (k_1) is very rapid. The reactions of Compound I and Compound II with aromatic donor molecules are slower; depending on the nature of the substrate, the k_2 and k_3 rate constants vary over 10^5-10^6 $M^{-1}s^{-1}$ and 10^3-10^5 $M^{-1}s^{-1}$, respectively. The reaction with ABTS is slightly more complex than with most other substrates; however, the general rule is that the k_3 is 10-20 times slower than k_2 for the ABTS scheme. Under most steady state conditions, k_3 is the rate limiting parameter (Smith et al, 1992).

Steady state peroxidase kinetics were performed using ABTS as the reducing substrate. Apparent values of $K_{m(ABTS)}$ and V_m/E were determined at a saturating concentration of hydrogen peroxide over the ABTS concentration range as described in section 2.22.1. The data were fitted to a PC-based graphics package (Enzfitter).

Steady-state and pre-steady state kinetics were also performed using ferrocyanide as the substrate. Ferrocyanide was chosen due to the fact that it undergoes a simple one-electron oxidation to ferricyanide without the production of free-radical intermediates. Although ferrocyanide is not a substrate of physiological significance for HRP, analysis of the k_3 rate data can reveal information about possible ionisation at the active site of HRP, which affects the kinetics of the ferrocyanide oxidation. Rate constants obtained by the steady-state method for ferrocyanide are in agreement with results obtained from studies of isolated reactions on the stopped flow apparatus. The steady state and pre-steady state kinetics for ferrocyanide were performed using purified (Section 2.23) and unpurified HRP. The steady state rate constants for the HRP-catalysed oxidation of ferrocyanide by hydrogen peroxide were compared with the rate constants obtained under pre-steady state conditions.

Pre-steady state kinetics were determined for a number of substrates i.e. ferrulic acid, indole-3-propionic acid (IPA) and ferrocyanide. Steady state kinetics can only give two pieces of information: 1) the K_m value, which may or may not be equivalent to the dissociation constant of the ES complex, and 2) the k_{cat} value which can be a microscopic rate constant but may also be a composite rate constant for several steps. In order to measure rate constants of individual steps on the reaction pathway and detect transient intermediates, it is necessary to measure the rate of approach to the steady state. It is during the time period when the steady state is set up that individual rate constants can be observed. Since the values of k_{cat} lie between 1 and 10^7 s^{-1}, measurements must be made on a time scale of 1 10^{-7}s. This requires a technique for rapid mixing and observation of the enzyme or substrate. Usually, the enzyme must be used in substrate-like amounts. Two methods of pre-steady state kinetic rate constant determination may be employed: the continuous flow method or the stopped-flow method. It was the latter that was used in these experiments.(Fig.7.7).

Fig. 7.7 Schematic Representation of Stopped Flow Apparatus



Conditions; Compound I was prepared by mixing equal volumes of 3.0mM HRP and 2.7mM H_2O_2. Compound I. On initiation both samples are mixed directly within the instrument. Experimental traces of ΔA_{424} against time during the reduction of Compound II by the various substrates are recorded.

7.4 Steady State Kinetics using ABTS as the Substrate

The steady state kinetics of native and modified HRP using ABTS as the substrate were determined as described in Section 2.22.1. The kinetics of ABTS were found to obey normal Michaelis-Menten kinetics for all the HRP forms. K_m and V_{max} values were calculated using Enzfitter. The results are summarised in Table 7.2 All Lineweaver-Burk plots were linear when inspected visually (K_m values are calculated from the Michaelis-Menten plots). Using a Molar extinction coefficient of $36mM^{-1}cm^{-1}$ (Gilfoyle et al., 1996), V_m/E values were determined at saturating concentrations of hydrogen peroxide. (See Table 7.2). The K_m and V_m/E values of native HRP (Table 7.2) were close to those reported by Gilfoyle et al. (1996) and Smith et al. (1992) i.e. 270μM and 810 s^{-1} respectively. The K_m values for both modified forms were increased (Table 7.2) with EG-NHS demonstrating the larger increase. EG-NHS exhibited 83% of the native HRP turnover. However, PA HRP exhibited an increase in its effectiveness towards ABTS as indicated by an increase in enzyme turnover (V_m/E).

Table 7.2 Steady state kinetic parameters for native and modified HRP using ABTS as Substrate

	K_m (μM)	Enzyme turnover (V_m/E) (s^{-1})
Native HRP	138 ± 14	751 ± 45
	158 ± 13	745 ± 50.5
EG-NHS HRP	207 ± 18	629 ± 55
	195 ± 19	581 ± 51
PA HRP	160 ± 14.5	990 ± 87
	195 ± 17	1008 ± 84.2

7.4.1 Steady State Kinetics using Ferrocyanide as the Substrate

The steady state kinetics of native and modified HRP using ferrocyanide as the substrate were determined as described in Section 2.23.2 according to the method of Hasinhoff and Dunford (1970). The kinetics were found to obey the Michaelis-Menten equation for the native and modified HRPs. The Lineweaver-Burk and Eadie-Hofstee plots (not shown) were linear. The $k_{3(app)}$ (M^{-1} s^{-1}) rate constants were determined from the slope of the reciprocal plot as described in Section 2.23.1 (Hassinhoff and Dunford 1970). Fig. 7.8 shows an example of the plots obtained for the native HRP. All values were the average of three determinations and were compared with the values obtained by pre-steady state methods. The purified and unpurified enzymes were also compared (Table 7.3). The results obtained for the native HRP were comparable to unpublished results determined by Dr. A.T. Smith (University of Sussex). In the case of native and PA HRPs, there was no significant difference between the values obtained for the purified and unpurified enzymes. However, in the case of the EG-NHS there was 10-fold difference between the two enzymes. The steady state kinetic values obtained for the native HRP were very close to those obtained in the pre-steady state experiments, 1.69×10^5 and 1.13×10^5 M^{-1} s^{-1} respectively. (See Fig. 7.11 for the results of the pre-steady state experiments).

Table 7.3 Rate Constants for the HRP Catalysed Oxidation of Ferrocyanide by Hydrogen Peroxide

Enzyme	$k_{3(app)}$ ($M^{-1}s^{-1}$) Steady state
Native HRP	$1.6 \pm 0.05 \times 10^5$
Native HRP (purified)	$1.69 \pm 0.06 \times 10^5$
EG-NHS HRP	$3.3 \pm 0.12 \times 10^6$
EG-NHS HRP (purified)	$2.24 \pm .021 \times 10^5$
PA HRP	$3.3 \pm 0.2 \times 10^5$
PA HRP (purified)	$3.03 \pm 0.1 \times 10^5$

Fig. 7.8

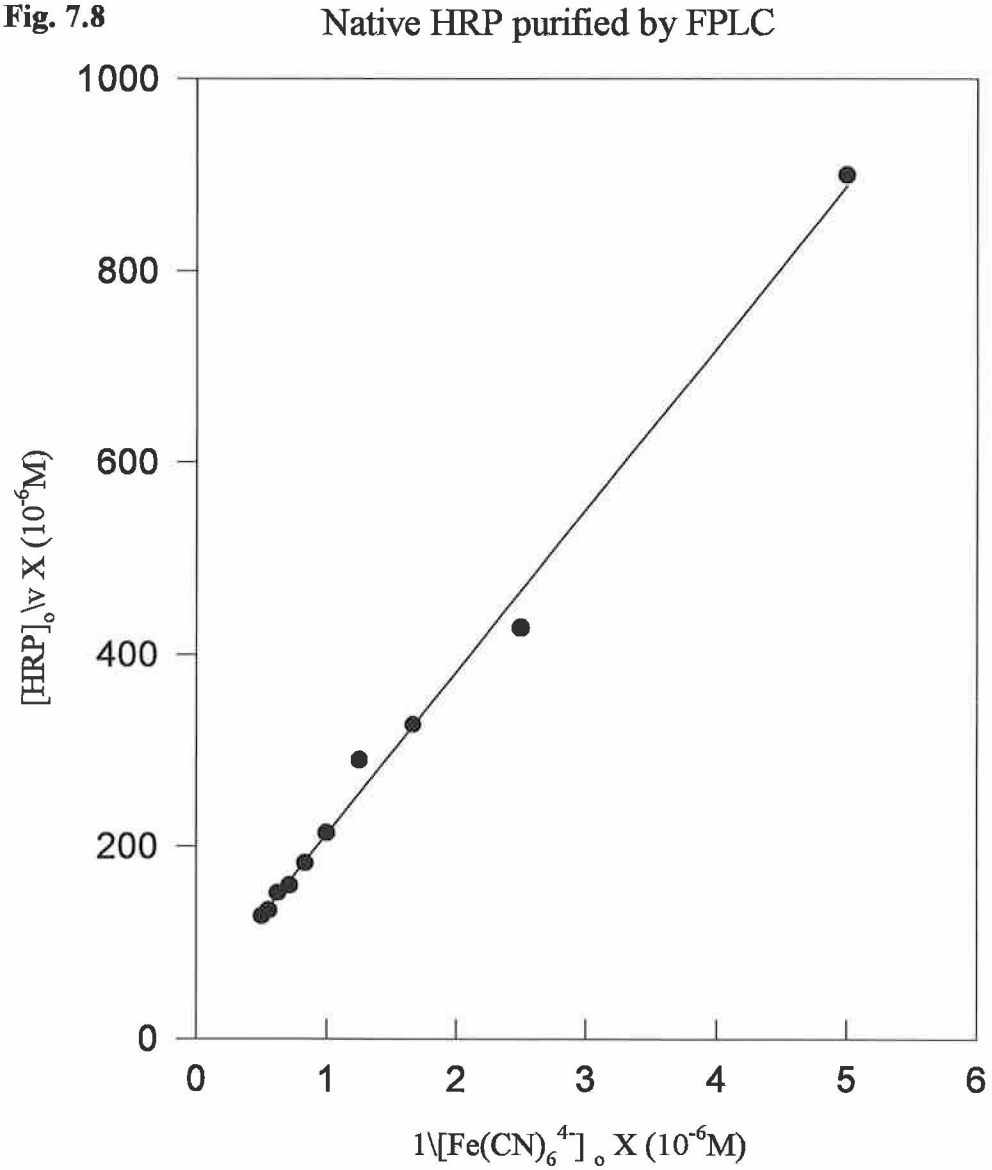


Fig. 7.8. Plot of $[HRP]_o/v$ vs. $1/[Fe(CN)_6^{4-}]_o$ in 10mM phosphate buffer, pH 7.0. The HRP concentration was kept constant at 0.9μM , and 1mM hydrogen peroxide was used.

7.5 Pre-Steady State Kinetics

7.5.1 Formation of Compound I

The method of Smith et al. (1993) was used as described in Section 2.22.3. The conditions for Compound I formation and pre-steady state kinetics had been optimised in the laboratory of Dr. A.T. Smith and his method was used without modification.

The standard method for the preparation of Compound I from HRP is the addition of peroxide in stoichiometric amounts. Compound I was therefore prepared by manual mixing of equal volumes of enzyme and hydrogen peroxide solutions of approximately equal concentrations. Any excess of Compound I in the reaction mixture will cause interference in the results. Therefore, the aim was to limit the formation of Compound I as much as possible. The decay of Compound I to Compound II is characterised by an isosbestic point at 395 nm together with a gain in intensity and a red shift of the Soret peak to 420 nm.

7.5.2 Formation and stability of Compound II

In general, the oxidation of reducing substrates by HRP Compound I is considerably faster (up to 1,000 times for some substrates) than by Compound II. Thus, given the classical peroxidase catalytic cycle (Fig 1.2), it should be possible to produce a relatively pure sample of Compound II by adding the appropriate concentration of reducing substrate to Compound I. Care must be taken with peroxidase stability and reducing substrate concentrations in order to avoid a sample containing a small amount of Compound II and a predominant amount of resting enzyme.

During the present work, a stable Compound II was formed by mixing enzyme: peroxide: reducing substrate (ferrocyanide) in the ratio 1: 0.9: 1.1. This ratio was achieved by trial and error. On addition of ferrocyanide to HRP Compound I, the change in the Soret peak with time indicated that a clean Compound II was formed. A Soret peak at 424 nm was obtained from this preparation (Fig. 7.9). An isosbestic point at approximately 405 nm during the decay curve (a result which is in agreement with the literature) is further evidence of preparation of Compound II.

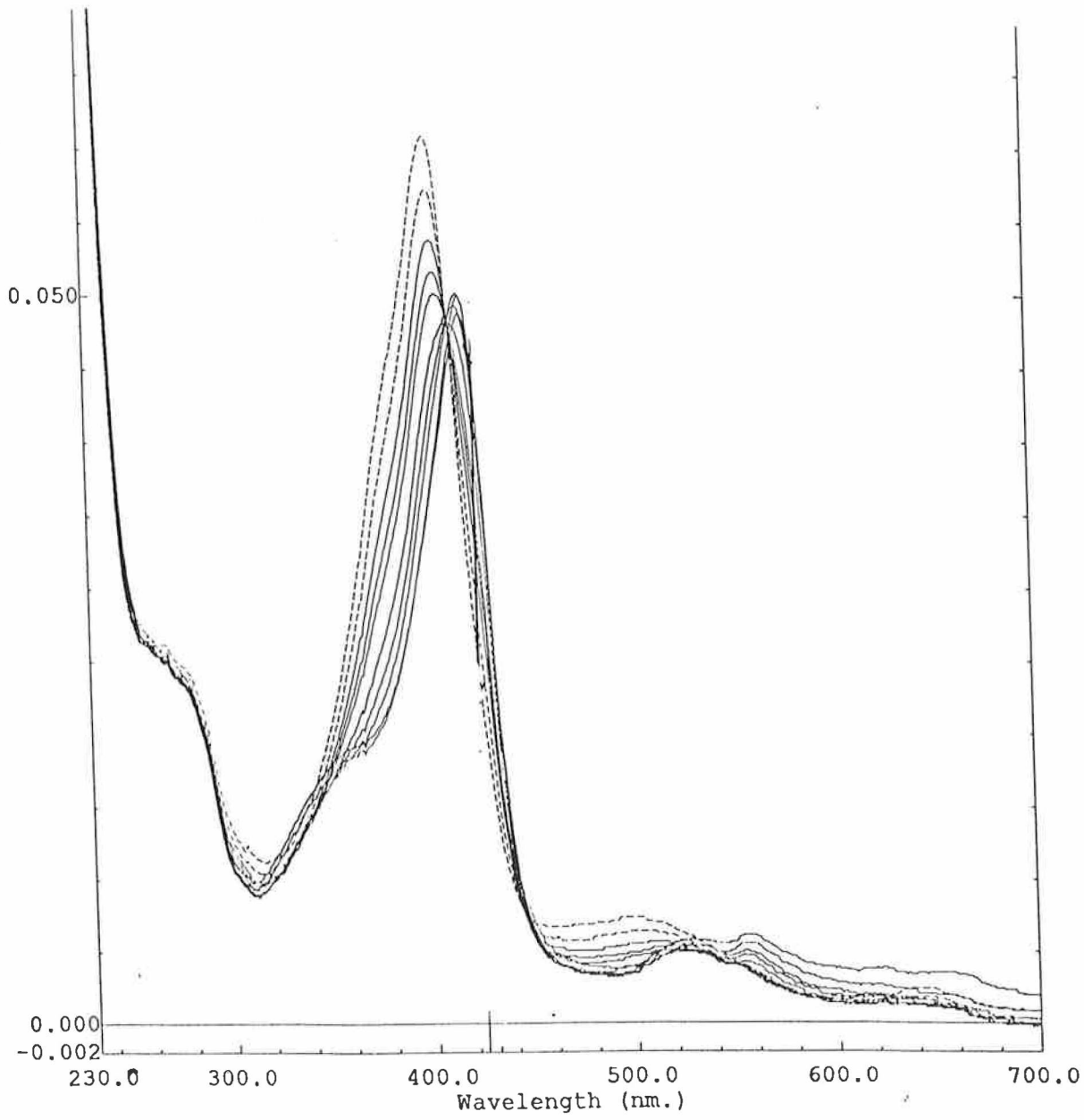
7.5.3 Estimation of k_3

For determination of k_3, Compound II was made by mixing molar equivalents of the substrates, A) ferrulic acid, B) indole 3-propionic acid and C) ferrocyanide with one mole of Compound I in the stopped flow apparatus as described in Section 2.23.2b

For the native enzyme and each derivative, values of k_{obs} were measured for the substrates in the range 4 μM -20μM (A and C) and 0.2 mM - 1.0 mM (B). Traces of ΔA_{424} against time during the reduction of Compound II by the various substrates were fitted to exponential functions.

For each enzyme with each substrate k_{obs} (average of 5 observations for each point) was plotted against substrate concentration. The slope of this graph was then k_3 with units of $M^{-1}s^{-1}$. (Fig. 7.10). Table 7.4 shows the k_3 values. Fig. 7.11 is a bar chart of the k_3 values obtained during this set of experiments (1-3), compared with the rate constants obtained in Dr. Smith's laboratory (4). It is clear that the values obtained for native HRP and derivatives were very similar to those of Dr. Smith and co-workers. Table 7.4 shows that the k_3 rate constant was faster for the two derivatives compared with the native for IPA and ferrulic acid. However, with ferrocyanide problems were encountered in the analysis of data and these are outlined in the legend to Table 7.4. As ferrocyanide is also a suitable substrate for use in steady state kinetics, the experiment was repeated employing steady state methodology (Section 7.4.2). The results of the steady state analysis, (Table 7.3) followed the above trend with the EG-NHS and PA HRPs displaying faster rate constants compared with the native.

Fig. 7.9 Formation of Compound II



Formation of Compound II: This was achieved by mixing enzyme: peroxide: reducing substrate (ferrocyanide) in the ratio 1:0.9:1.1. The absorption spectrum was scanned at 5 min intervals in rapid mode from 230nm to 700nm.

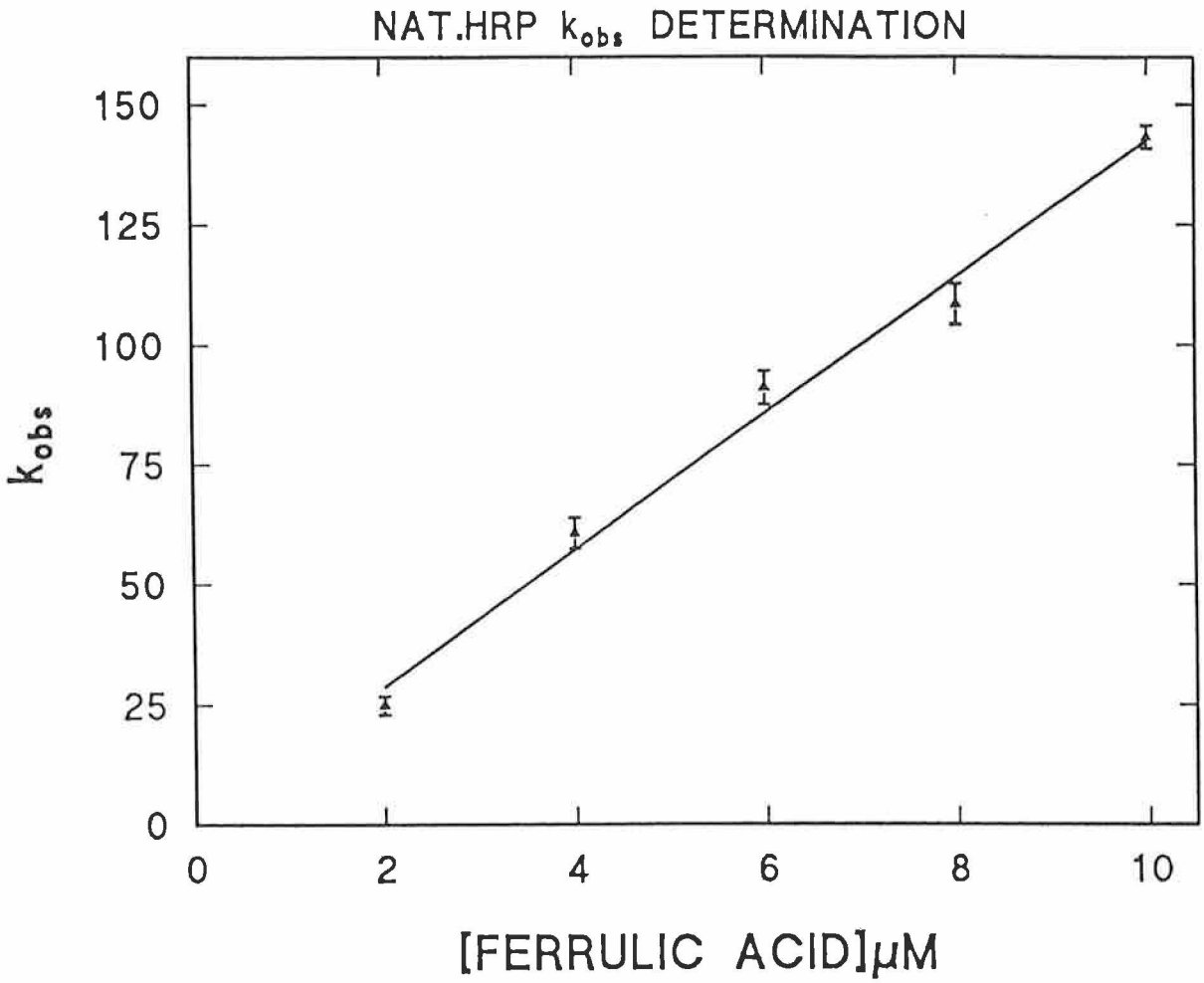


Fig. 7.10 Plot of k_{obs} for the Reaction of Native HRP with Ferrulic Acid to form Compound III in 10mM phosphate buffer, pH 7.0 at 25.0 ± 0.2 °C as a function of Ferrulic Acid Concentration. Each point is the average of three determinations with calculated standard deviations represented by error bars

Table 7.4 Pre-Steady State kinetic Data Obtained with H_2O_2 and Three Reducing Substrates in 10mM Phosphate Buffer, pH 7.0 at 25 °C.

Enzyme	Substrate	k_3 [$M^{-1}s^{-1}$][a]
Native HRP	Ferrulic acid	$11.6 \pm 0.39 \times 10^6$
	IPA*	556.00 ± 17.00
	Ferrocyanide[1]	$1.13 \pm 0.126 \times 10^5$
EG-NHS HRP	Ferrulic acid	$14.44 \pm 0.7 \times 10^6$
	IPA*	829.00 ± 2.2
	Ferrocyanide[2]	$1.06 \pm 0.109 \times 10^4$
PA HRP	Ferrulic acid	$14.21 \pm 0.85 \times 10^6$
	IPA*	757.00 ± 43.00
	Ferrulic acid[3]	$0.75 \pm .098 \times 10^5$

[a] The k_3 rate constants were calculated by a least squares regression procedure from slopes of the graphs (e.g. Fig.7.10). with errors calculated as one standard deviation.

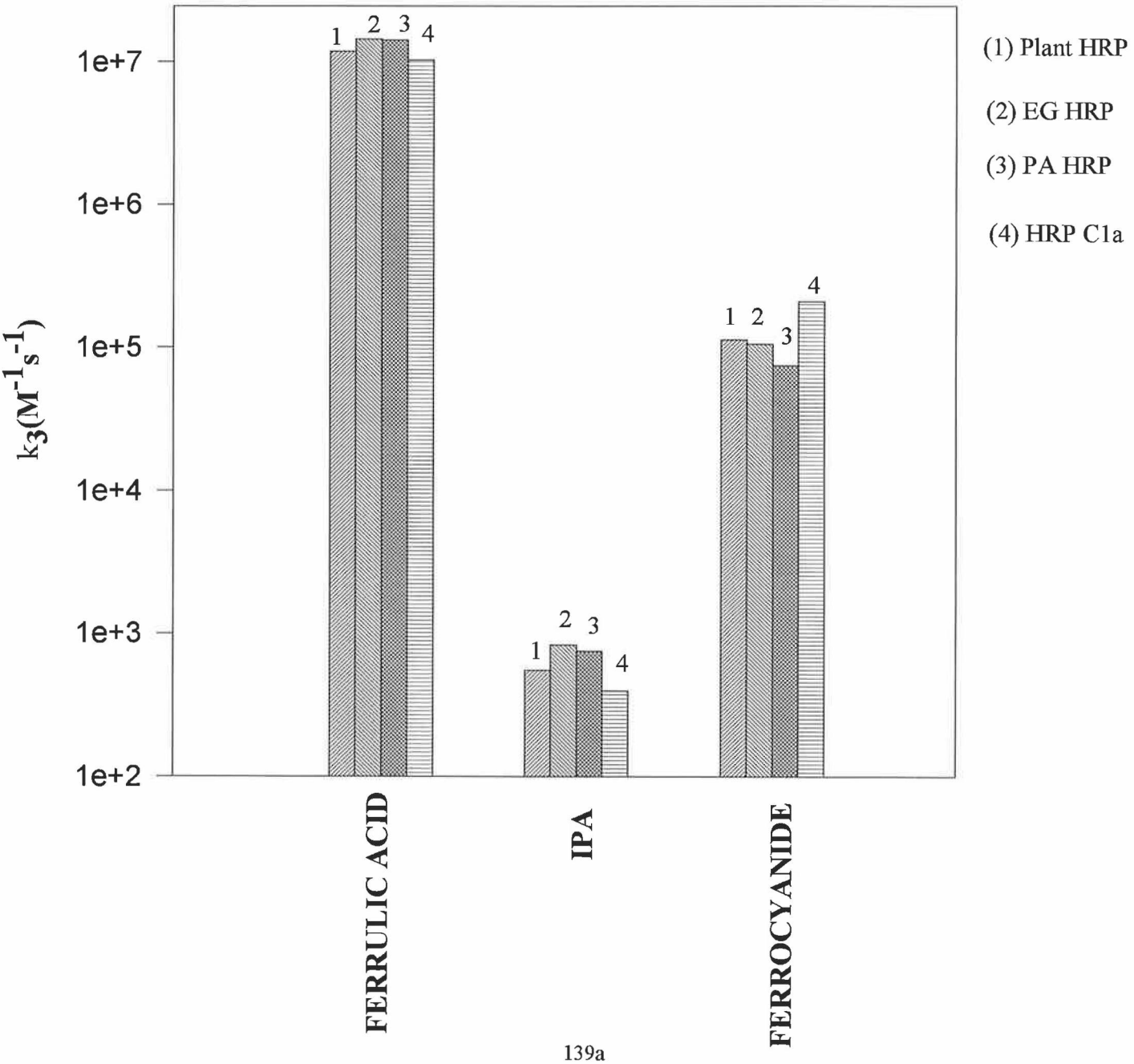
* Indole 3-propionic acid

1- mean of only three values

2- mean of only four values

3- appeared to be a double exponential function.

Fig. 7.11 **Steady State kinetics of Native and Modified HRP Compared with Recombinant HRP**



CHAPTER 8

WASTEWATER CLEAN UP

8.0 Waste Water Clean-up

8.1 Introduction

When water is in abundance, then it is very easy to take it for granted. This is specially so in this country, which is not only an island but boasts a multitude of lakes. It is said that their are 365 lakes in Co. Cavan (one for every day of the year). An uncontaminated source of water is essential for every day life. This is very evident if you look at the way towns have grown up around water sources. That was during a time when finding a source of water was all-important and the quality of the water was taken for granted. The quantity of the pollutants contaminating the water was relatively small and those present were often removed through natural processes. It was also impossible to detect pollutants that were not visible to the naked eye, therefore what could not be seen was not known to be present. However, increasing population, growing industry and rapidly developing technology since the industrial revolution have continued to test Nature's capacity for maintaining clean water. Increased water use and wastewater discharge have added impurities to water which overload natural cleaning processes, either because of the amount or the chemical complexity of the impurities. An example of this was evident with the Proctor and Gamble spill in Nenagh in the summer of 1996. Due to the accidental spillage into the river, the town was without water for many days and virtually came to a standstill. We are, therefore, forced to turn to technology to protect our water supplies.

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" is used in this context to include not only the parent phenol (C_6H_5OH) but also an array of organic compounds containing one or more hydroxyl (OH) groups attached to an aromatic ring. The adverse effects of phenols in waste water are well known. Such aromatic hydroxy compounds can be toxic at elevated levels and are known or suspected carcinogens (Industrial Wastewater Report, 1990). As little as 0.005mg L^{-1} of phenol is enough to cause an unpleasant taste and odour to drinking water when combined with chlorine to form chlorophenols. Thus, it is of great practical significance to remove such chemicals from water or industrial effluents.

A number of methods are currently available to remove phenolic compounds from wastewater. These include microbial degradation, chemical oxidation (agents such as hydrogen peroxide, chlorine dioxide and ozone have been used), adsorption onto activated carbon, incineration, solvent extraction and irradiation (Spiker et al., 1992; Throop, 1977.) The choice is as much dependent on cost as on any other factor.

A method for the removal of toxic aromatics from wastewater using horseradish peroxidase (HRP) was first proposed by Klibanov et al. (1980). HRP catalyses the oxidation of a variety of phenols and aromatic amines in the presence of H_2O_2, generating phenoxy radicals (Yamazaki et al, 1960). These free radicals diffuse from the active centre of the enzyme into solution where they attack similar or dissimilar molecular species to form water-insoluble polyaromatic products. The free radicals are non-specific and so react with both good and poor peroxidase substrates. This non-specific interactiveness of the enzyme-generated free radicals has a practical value, since the pollutant population of a wastewater system is often a complex mixture of chemicals with varying degrees of susceptibility to peroxidase attack (Neidleman, 1984). Klibanov et al, (1983) estimated that one molecule of peroxidase destroys approximately 10^3 molecules of phenol in its lifetime. Furthermore, two free radicals are generated for every molecule of peroxide consumed (Reaction 8.1).

$$H_2O_2 + 2AH_2 \xrightarrow{HRP} 2\,AH^{\bullet} + 2H_2O \qquad (8.1)$$

This enzymatic approach is suitable for the treatment of wastewaters containing aromatic contaminants. The most significant feature of these, apparently less toxic compounds is that they are nearly water-insoluble (Nicell, 1994). Therefore, this simple peroxidase-catalysed reaction, which transforms water-soluble toxic phenols into water-insoluble ones, could be used generally for the removal of pollutants from water. This is because simple filtration or sedimentation procedures could then easily separate the insoluble chemicals from the water (Alberti & klibanov 1983; Nicell et al., 1992). This enzymatic approach has many advantages over conventional procedures (Klibanov et al., 1983; Nicell et al., 1992).

Currently methods such as solvent extraction and adsorption onto activated carbon are effective, but have a number of shortcomings; e.g., high cost, incomplete purification, formation of hazardous by-products and applicability to only a limited concentration range. Due to inactivation, large amounts of enzyme are required to achieve a high degree of phenol removal. This has limited its use to date in industrial situations (Klibanov & Morris, 1981). Klibanov et al. (1983) suggested that this inactivation was most likely a result of interactions of phenoxy radicals with the enzyme's active site. In this situation it would be nearly impossible to reduce the amount of enzyme required. Nakamoto & machidia (1992) have pointed out that enzyme inactivation may in fact be due to adsorption of enzyme molecules onto the end-product polymer, limiting diffusion of the substrate to the active centre. Other strategies, such as the use of additives like polyethylene glycol (PEG) and gelatin, are known to exert a protective effect on HRP by suppressing enzyme adsorption without changing the reaction stoichiometry between H_2O_2 and phenol (Wu et al., 1993). It was noted that the higher the PEG concentration, the greater the degree of protection afforded. Immobilisation techniques also offer a solution. Here the enzyme is attached to a suitable insoluble carrier (perhaps via a periodate linkage) and inactivation may therefore be delayed through prevention of unfolding (Davies & Burns1990; Siddique et al., 1993). Nicell et al. (1991), however, pointed out problems whereby mass transfer limitations evolve, resulting in the formation of reaction products on the carrier matrix and reducing the capacity for enzyme-catalysed polymerisation reactions.

Temperatures in excess of 50 °C result in a significant reduction in the peroxidase-catalysed decolorisation of bleached kraft mill effluent. Kraft effluent is the contamination produced by pulp and paper industries, principally in the alkaline extraction stage of wood -pulp bleaching , which accounts for over 50% of the colour load (Pace & Jurasek, 1984). Hydrogen peroxide is an efficient bleaching agent in its own right, as well as functioning as a substrate in the HRP catalytic cycle. However, the bleaching capacity of H_2O_2 has been increased when incorporated into a HRP-peroxide system. Davies & Burns (1990) reported that the most efficient decolorisation took place at H_2O_2 : HRP ratios of less than 3 mM : 1 unit of HRP. Horseradish peroxidase removes colour from pulp mill effluents but rapid and irreversible inactivation takes place. Entrapment in alginate beads

improves the decolorisation process by several-fold (Ferrer et al., 1991). Davies et al. (1990) also reported that entrapment of HRP in alginate beads improved decolorisation by up to 36-fold in some cases. However, this method is unsuitable for continuous use as the enzyme is released into solution too rapidly.

Synthetic dyes are used extensively for textile dyeing, paper printing, colour photography and as additives in petroleum products (Youngless et al., 1985). Over 7×10^5 tons of these dyes are produced annually and 10-15% of these are lost in effluent. These dyes are resistant to microbial degradation and are, therefore, not easily removed in wastewater treatment plants (Nigam Marchant, 1995). Welinder et al (1993) have patented a peroxidase variant, recombinant *Coprinus* Peroxidase (CIP), with an improved stability towards hydrogen peroxide. Thus, higher concentrations of peroxide can be used in the bleaching system, resulting in improved decolorisation, one can also prevent surplus dyes leaching from coloured fabrics during normal washing procedures. High temperatures, occurring during normal washing conditions, are known to inactivate HRP and it would be desirable, therefore, to have a peroxidase that could withstand elevated temperatures.

HRP loses catalytic activity at elevated temperatures due to unfolding of the protein backbone. Often, subsequent inactivation is caused by the destruction of one or two "weak points" such as fragile peptide bonds or easily-oxidisable functional groups (Mozhaev et al, 1982). However, thermostability and solvent stability of HRP has been significantly increased by chemical modification with a range of amino-specific bis-succinimides. (Ryan et al, 1994; Miland et al., 1996) and with phthalic anhydride modification (see chapter 4).

The purpose of the following work is to investigate the effect of phthalic anhydride modification on (a) The HRP-catalysed removal of parent phenol and other phenolic compounds at high temperatures and to compare the performance of the PA-HRP with that of EG-NHS HRP as reported by Miland et al. (1996), (b) (PA-HRP and EG-HRP) on a range of dyes in the presence of different accelerators.

8.2 HRP-Catalysed Oxidation of Phenol at 37 °C

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. A number of conditions have been optimised previously (Miland, 1996) for phenol removal. These include:

(a) Activity of HRP

(b) Reaction time

(c) Effect of pH

(d) Temperature

(e) Concentration of succinimide

(f) Hydrogen peroxide concentration

With this in mind an intensive study was not undertaken. Instead it was decided to use the conditions optimised by Miland (1996) to compare the removal efficiency of the native HRP with the two modified forms under defined experimental conditions. The phenol removal was carried out as described in Section 2.24. Upon addition of HRP (final activity of 0.3 U ml^{-1}) 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. This is followed by separation of a brown precipitate which can easily be removed by centrifugation to give a colourless solution. To determine the removal efficiency by this treatment, a solution of phenol is analysed before and after the addition of the HRP system (HRP-H_2O_2). Under these conditions, treatment for 20 minutes at 37 °C resulted in excess of 80% removal of parent phenol for native and the two modified forms of HRP. The removal efficiency for chlorophenol was even greater; 20 minutes reaction time resulted in $\geq$ 95% removal in all cases (Table 8.1). A mixture of the two phenols in a 1:1 ratio gave slightly lower removal efficiencies (approximately 71%) for all three forms. Removal efficiencies were greatest when the reacting solutions were agitated rather than being left still. It should be pointed out that treatment with either peroxidase or peroxide alone did not result in any aromatic removal from aqueous solution. At 37 °C, very little difference was noted in the removal efficiencies of the native compared with the modified forms of HRP (Fig.8.1).

8.2.1 HRP Catalysed Oxidation of Phenol at 70 °C

High temperature experiments were carried out as for those at 37 °C (Section 2.24.1). A ten minute preincubation period at 70 °C prior to starting the reaction was found to be important to ensure that all these reagents reached the reaction temperature. The removal efficiencies at 70 °C were less than half those noted at 37 °C. In the case of phenol, the removal efficiencies were 32.5%, 41% and 45.5%. for the native, EG and PA HRP respectively. For chlorophenol the values were 32.5%, 41% and 42% and for the mixture removal efficiencies were 24.7%, 36% and 36% for the native, EG and PA HRP respectively (Table 8.2). Unlike the experiments at 37 °C, the high-temperature experiments indicated differences in performance between the native and the modified forms. The modified enzymes were up to 50% more efficient at phenol removal than the native at this elevated temperature (Fig. 8.1-8.3).

Table 8.1 % Removal Efficiencies of Native and Modified HRP at 37 °C

	Phenol (%)	4-Chlorophenol (%)	Mixture (%)*
Native HRP	85.6 ± 8.0	93 ± 2.1	72 ± 6.3
EG-NHS HRP	81.5 ± 6.5	95 ± 1.6	71.5 ± 5.9
PA HRP	82.3 ± 7.9	95 ± 8.2	71.3 ± 6.9

* mixture indicates a 1:1 ratio of phenol : 4-chlorophenol

Fig. 8.1 **% Phenol removed in 20 minutes**

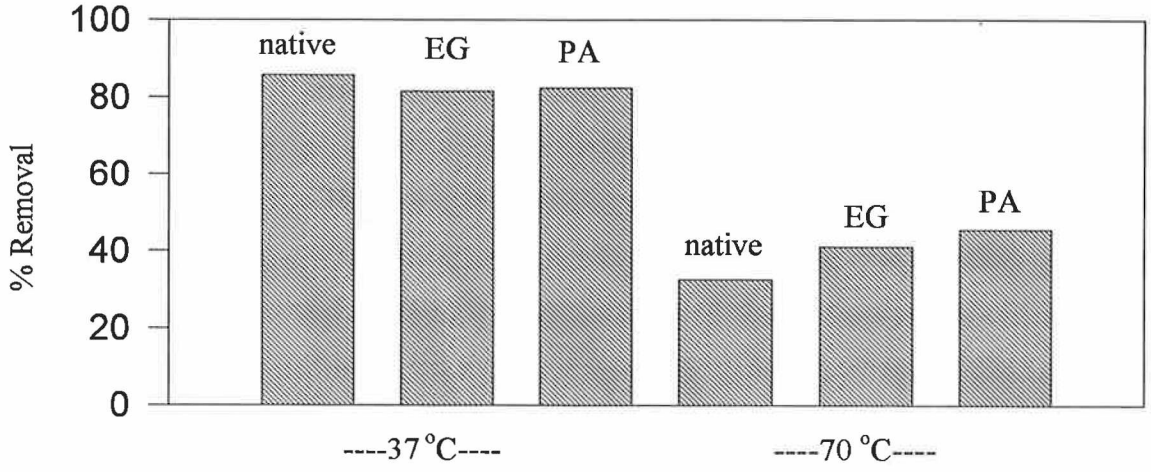


Fig. 8.2 **% Chlorophenol removed in 20 minutes**

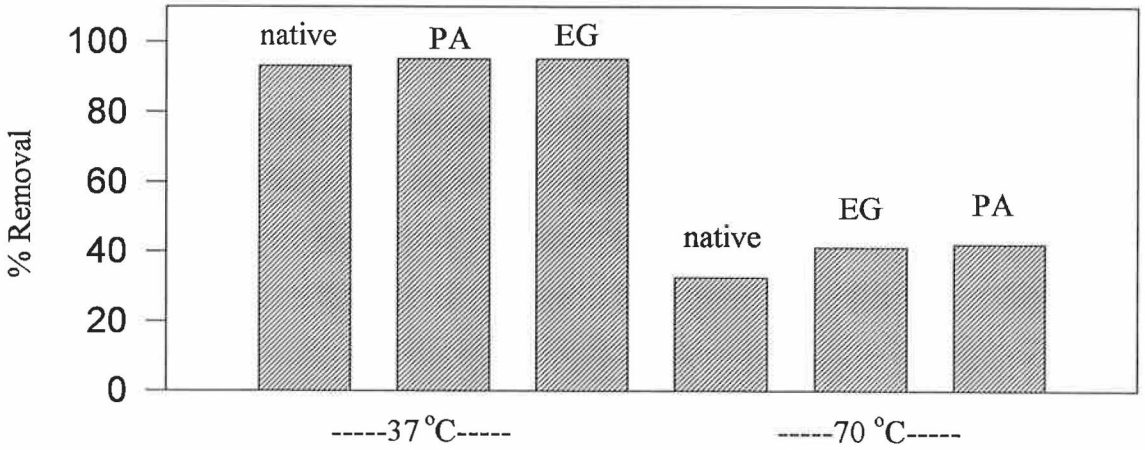


Fig. 8.3 **% Mixture removed in 20 minutes**

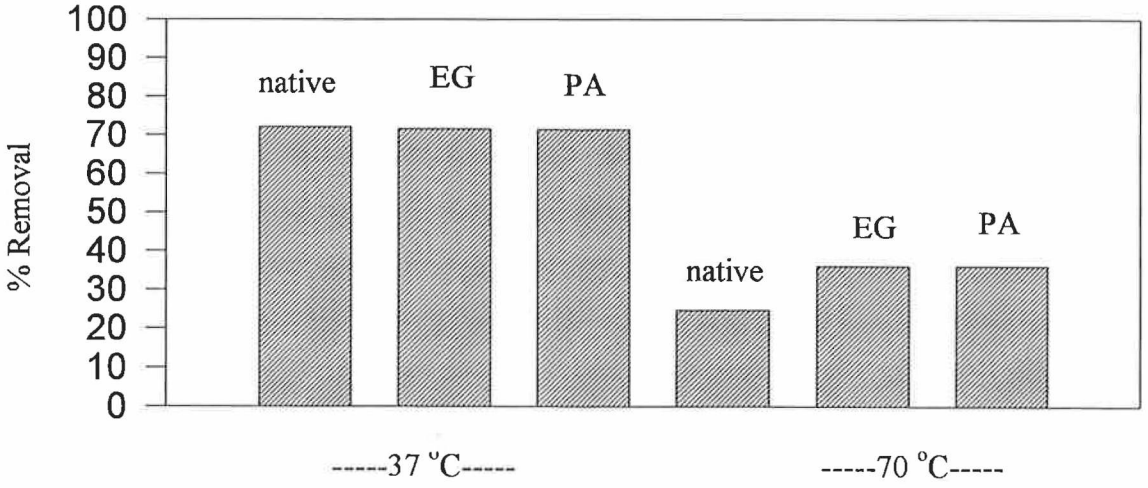


Table 8.2 % Removal Efficiencies of Native and Modified HRP at 70 °C

	Phenol (%)	4-Chlorophenol (%)	Mixture (%)*
Native HRP	32.5 ± 0.7	32.5 ± 3.0	24.7 ± 0.8
EG-NHS HRP	41 ± 3.1	41 ± 1.9	36 ± 1.2
PA HRP	45.5 ± 1.5	42 ± 0.9	36 ± 1.4

* mixture indicates a 1:1 ratio of phenol : 4-chlorophenol

8.3 Bleaching of Dyes

8.3.1 Optimisation of Assay

The dyes were incubated in the range 4-8μM with increasing concentrations of HRP and assayed for residual absorbance at λ max compared with the untreated dye control at fixed times. The concentrations of H_2O_2 and accelerator were identical in all experiments, i.e. 200μM and 50μM respectively. The percentage of colour removed was determined by measuring the decrease in absorbance at the appropriate wavelength. The absorbance of the control sample, i.e. containing everything except HRP, was taken as 100% colour. 45nM HRP incubated for 10 minutes in the reaction mixture was determined give the most efficient colour removal (See Fig. 8.4).

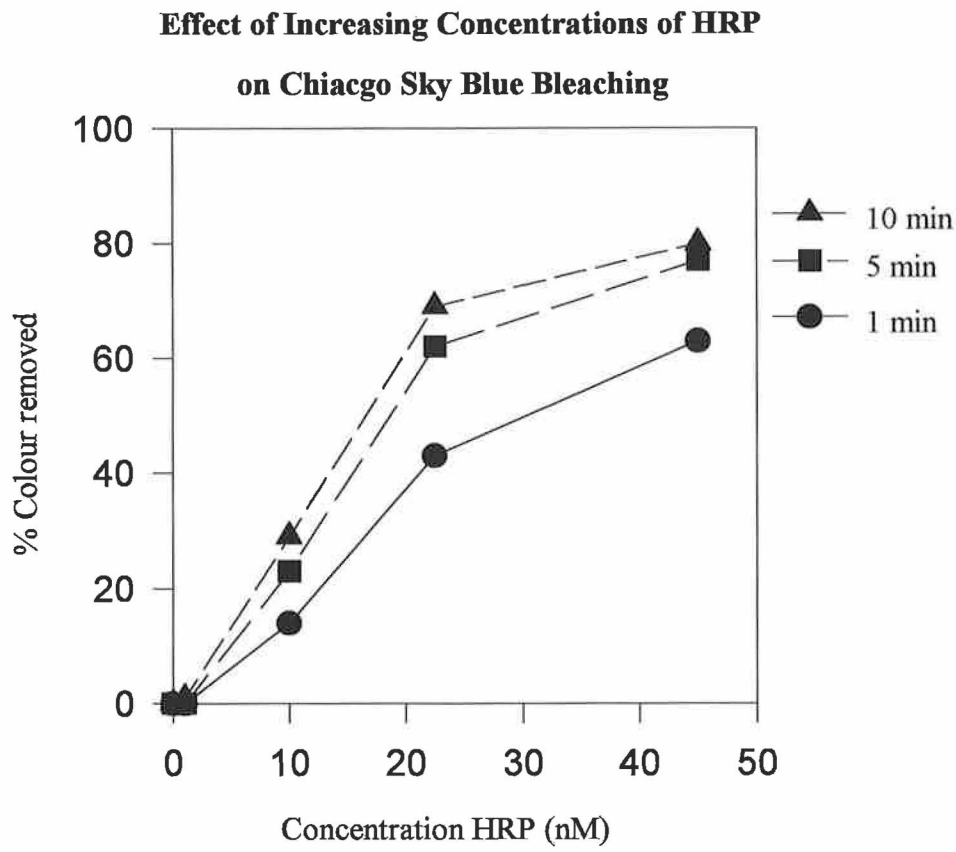
8.3.2 Bleaching Effect of Native and Modified HRP at 37 °C

Each dye was scanned initially to determine the wavelength of maximum absorption (Fig. 8.5). Having previously determined the optimum HRP concentration for maximum bleaching (Section 8.3.1), the reaction mixtures could then be inspected visually for bleaching prior to spectroscopic analysis. On addition of HRP to the test system dye : accelerator : H_2O_2, in the ratio 4-8 : 50 : 200 the dye colour disappeared. In the case of the blue dyes, complete bleaching was achieved in most cases. However, the red and orange

dyes merely faded to pale orange and complete colour removal was rarely achieved. The conditions outlined in Section 2.23.1 were used for both the native and the modified HRPs. Direct comparisons were made between the native and modified enzymes under identical conditions.

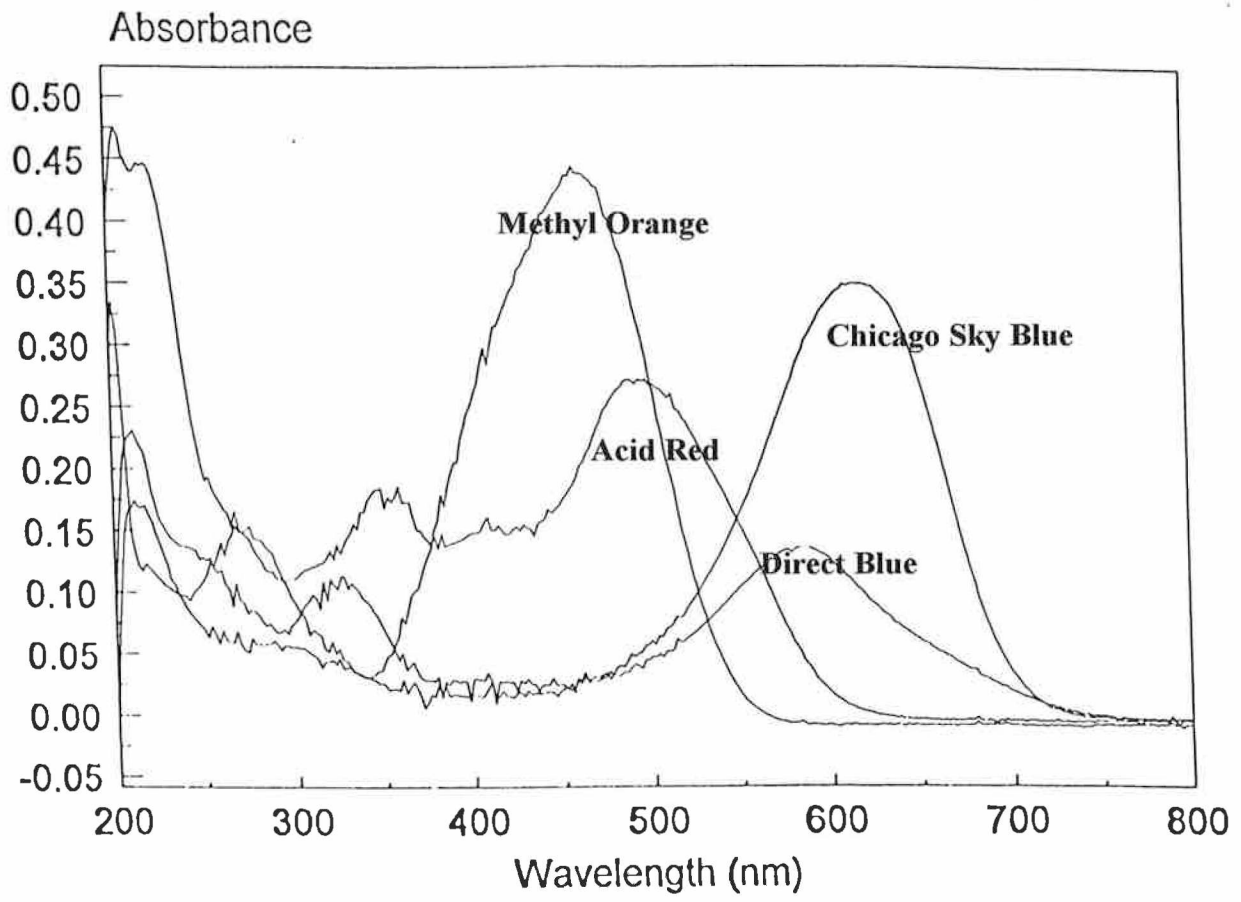
The samples were incubated at 37 °C for 10 mins and scanned continuously (Fig. 8.6 shows an example of scans obtained). The percentage of colour removed was determined by measurement of the absorbance before, and 10 mins after, the addition of HRP at the λ max suitable for the particular dye (Fig. 8.6). Each of the four dyes, Acid Red 151, Chicago Sky Blue 6B, Direct Blue 71 and Methyl Orange were tested in the presence of the four accelerators, 4-hydroxycinnamic acid, 7-hydroxycoumarin, 4-hydroxybenzsulfonic acid and vanillin (Table 8.3). It was found that the bleaching capacity of HRP varied from dye to dye and was also dependent on the accelerator used. The modified forms were as efficient bleaching agents as the native HRP at 37 °C. The type of accelerator employed appeared to determine to some extent the degree of bleaching. For example, the percentage of colour removed from Acid Red varied from 30% in the presence of 4-hydroxycinnamic acid to 73% using vanillin as the accelerator. Use of vanillin as accelerator gave consistently better bleaching than did any of the other accelerators.

Fig. 8.4



Conditions: 8μM Chicago Sky Blue, 50μM 4-hydroxybenzesulfonic acid, 200μM hydrogen peroxide. All spectroscopic determinations were carried out at room temperature.

Fig. 8.5 **Scans of the Dyes used in the Bleaching Experiments**



Conditions: 4μM of each dye scanned from 200-800nm. Peak at 465nm =Methyl Orange, 490nm =Acid Red, 580nm = Direct Blue, 618nm =Chicago Sky Blue

Table 8.3 Bleaching Effect of Native and Modified HRP in the Presence of Different Accelerators (37 °C)

	HRP	Direct Blue	Chicago-Sky Blue	Acid Red	Methyl Orange
7-hydroxy-cinnamic acid	Nat	62%	78%	52%	67%
	EG	61%	79%	37%	56%
	PA	59%	76%	29%	55%
7-hydroxybenz-sulfonic acid	Nat	70%	81%	24%	57%
	EG	70%	79%	22%	50%
	PA	69%	73%	28%	36%
7-hydroxycoumarin	Nat	62%	86%	84%	28%
	EG	51%	81%	83%	27%
	PA	61%	84%	82%	21%
vanillin	Nat	73%	84%	64%	63%
	EG	71%	84%	63%	51%
	PA	70%	84%	67%	52%

All reactions were carried out for 10 min at 37 °C,

%, indicates the percentage of colour removed in 10 minutes,

Conditions: HRP 45μM, H_2O_2 200μM, Dye 4-8μM, Accelerator 50μM in reaction volume of 1ml.

8.3.3 Bleaching Effect at Elevated Temperature (65 °C)

The bleaching of the dyes at high temperatures was determined as described in Section 2.23.2. Essentially, the conditions were the same as the low temperature studies. All the reagents except HRP were brought to the required temperature prior to initiation of the bleaching process by addition of enzyme. For the elevated temperature studies it was noted that the modified peroxidases were more efficient bleaching agents than the native in the presence of vanillin and 7-hydroxycinnamic acid (Table 8.4). The modified HRP performed as well as the native when used in conjunction with the other accelerators. It was noted that the extent of bleaching was greatly reduced at the elevated temperatures compared with the low temperature studies. However, in the case of 7-hydroxycinnamic acid (Table 8.4) the percentage of colour removed at 65 °C was actually enhanced. It is possible that the increased temperature enhanced the oxidising capabilities of the accelerators.

Table 8.4 Bleaching Effect of Native and Modified HRP in the Presence of Two Different Accelerators (65 °C)

	Vanillin/7-Hydro. Chicago-Sky Blue	Vanillin/7-Hydro. Direct Blue	Vanillin/7-Hydro. Acid Red	Vanillin/7-hydro. Methyl Orange
Native	65.7% / 99%	8.2% /86%	65% / 90%	n.c.r / 50%
EG	71% / 100%	11.2% / 98.5%	69% / 89%	1.6% / 56%
PA	73% / 100%	10% / 97.3%	66% / 84%	1.6% / 56%

All reaction were carried out for 10 min at 37 °C,
%, indicates the percentage of colour removed in 10 minutes
n.c.r = no colour removal; 7-hydro. = 7-Hydroxycinnamic Acid.
Conditions: HRP 45μM, H_2O_2 200μM, Dye 4-8μM, Accelerator 50μM in reaction volume of 1ml.

8.3.4 Bleaching Effect in the Presence of Solvents

Vanillin was chosen as accelerator for the bleaching reactions in the presence of solvents. The reaction mixtures were incubated using the conditions outlined in Section 2.23.3. Dioxane and DMF were used in this set of experiments. The percentage of colour removed was greatly reduced in the presence of both dioxane and DMF. No colour was removed from Methyl Orange. On incubation with dioxane for 10 min, the modified HRP consistently removed more colour from all dyes tested with the exception of Methyl Orange (Table 8.5). Colour removal in the presence of DMF was not as good as with dioxane, the exception being the colour removal from Chicago Sky Blue, where up to 80% of the colour was removed (Table 8.5).

Table 8.5 Bleaching Effect of Native and modified HRP using Vanillin as Accelerator in the Presence of Dioxane and DMF

	Solvent	Methyl Orange	Acid Red	Direct Blue	Chicago-Sky Blue
Native	Dioxane	n.c.r	11%	8%	62%
EG	Dioxane	n.c.r	11.1%	10%	67%
PA	Dioxane	n.c.r	21%	13%	63%
Native	DMF	n.c.r	n.c.r	4%	57%
EG	DMF	n.c.r	5%	4%	87%
PA	DMF	n.c.r	n.c.r	4%	56%

All figures denote the percentage of colour removed in 10 min.
n.c.r = no colour removal.
Conditions: HRP 45μM, H_2O_2 200μM, Dye 4-8μM, Accelerator 50μM.

8.3.5 Bleaching Effect in the Presence of Immobilised HRP

The HRP was immobilised as described in Section 2.20. It was found that approximately 70% of the HRP was immobilised. The HRP concentration was determined as 1mg ml^{-1} using the BCA assay (Section 2.3.1.1). The TMB assay (Section 2.2.1) was used to assay for relative activity and it was found that 1:1000 and 1:100 dilutions of the immobilised native and modified HRP respectively, gave absorbance values that corresponded to approximately 5μg L^{-1} when compared with soluble HRP assayed under similar conditions.

The immobilised HRP was incubated with Chicago Sky Blue and vanillin as accelerator (using the conditions outlined for the unmodified HRP in section 8.3.2) and was scanned every 1.5 minutes for 15 minutes. The performance of immobilised native and EG-HRP compared favourably with the results obtained for the unimmobilised HRP with > 80% of the colour removed after 4 minutes at room temperature. The PA-HRP was not as efficient at removing the colour. After 15 minutes, approximately 50% of the colour remained. The immobilised HRP has been shown previously to be more stable at 65 °C than soluble HRP. However, these bleaching experiments were carried out at room temperature. (Fig 8.6).

8.3.6 Effect of Successive Additions of Dye on Bleaching

The percentage of colour removed on successive additions of dye and hydrogen peroxide was determined as described in Section 2.27.5. It was found that the native HRP continued to remove > 60% of the colour from Chicago Sky Blue for up to 5 cycles. The modified HRPs performed well with the EG proving to be capable of removing > 18% of the colour and the PA 13% after 5 cycles. Table 8.6 illustrates the percentage of colour removed after 10 minutes after each addition of 20 μl of dye (stock 400 μM).

8.3.7 Effect of Successive Additions of Dye on Bleaching with Immobilised HRP

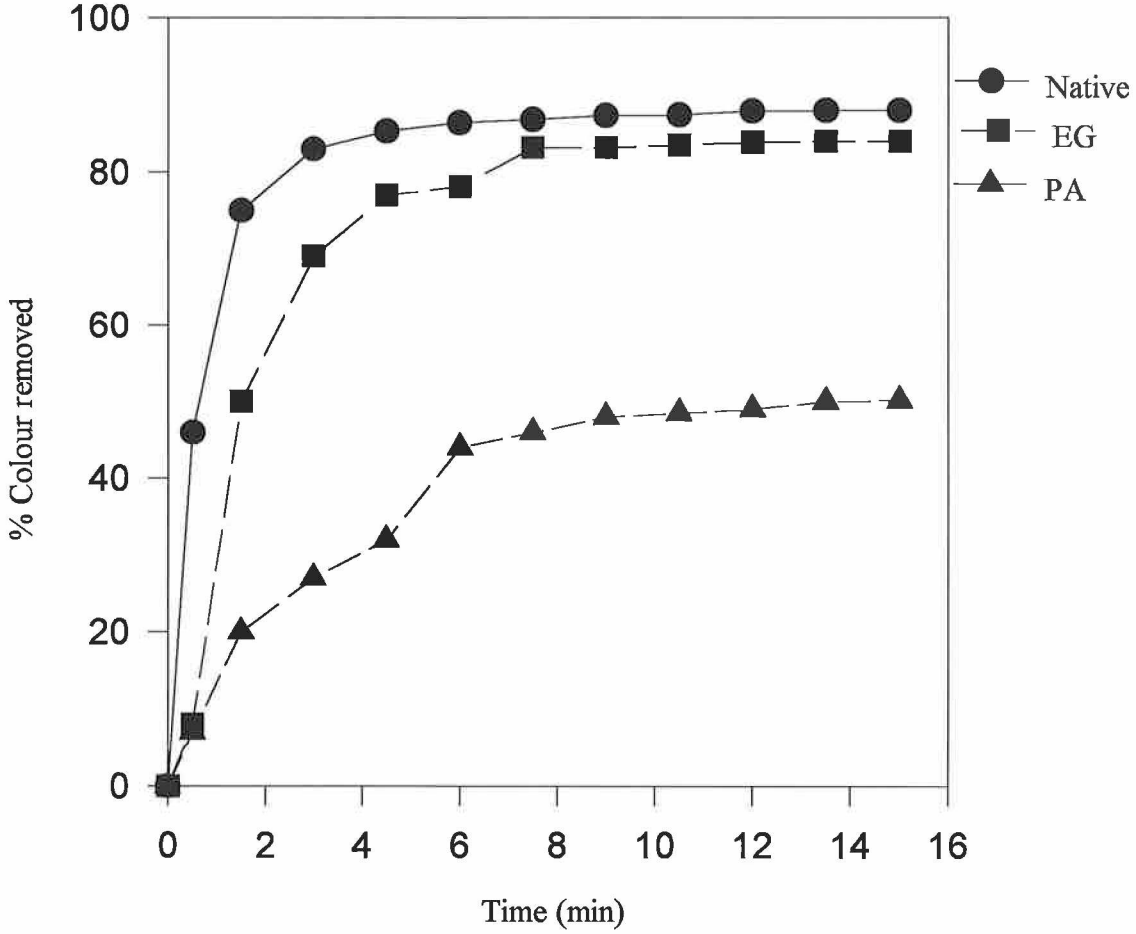
The percentage of colour removed on successive additions of dye and hydrogen peroxide was determined as described in Section 2.27.5. It was found that the native immobilised HRP continued to remove > 90% of the colour from Chicago Sky Blue for up to 5 cycles. The immobilised modified HRPs performed well, with the EG proving capable of removing > 62% of the colour and the PA as much as 43%. At the end of 5 cycles of bleaching the immobilised HRP both native and modified continued to consistently remove more colour from the dye than the free form. Table 8.6, illustrates the percentage of colour removed after 10 minutes after each addition of 20 µl of dye (stock 400 µM). The percentage of colour removed using the immobilised even after 5 cycles of bleaching was high compared with the soluble enzyme.

Table 8.6 Bleaching Effect on Chicago Sky Blue using Vanillin as Accelerator upon Successive Addition of Dye

Cycles	Control	Native HRP Imm./Free.	EG HRP Imm./Free	PA HRP Imm./Free
One	100%	86% / 79%	82% /70%	70% /44%
Two	100%	89% /69%	66% /56%	59% /22%
Three	100%	90% /67%	68% /39%	53% /18%
Four	100%	90% /68%	67% /28%	48% /18%
Five	100%	90% /64%	62% /18%	43% /13%

All figures denote the percentage of colour removed in 10 min at room temperature.
Imm. Immobilised HRP, Free = non immobilised HRP
Conditions: reaction mixture [8µM dye, 50µM Vanillin, 200µM hydrogen peroxide, HRP concentration (45nM for free HRP in a total volume of 1ml), Imm. Concentration (1:1000 and 1:100 for native and modified)]
Cycles: 20µl dye (400µM stock), 50µl hydrogen peroxide (200µM stock) also added to the reaction mixture minus HRP (i.e. control sample.)

Fig. 8.7 **Effect of Bleaching on Chicago Sky Blue using Immobilised HRP with Vanillin**



CHAPTER 9

DISCUSSION AND CONCLUSION

9.0 DISCUSSION

9.1 Studies on Native HRP

9.1.1 TMB Assay

The TMB assay used to determine HRP's activity, based on the method of Bos et al. (1981) and Gerber et al (1985), was optimised with respect to concentration. This had been previously optimised by Miland et al. (1996) and a concentration of 80 μg L^{-1} was deemed to be acceptable. The results of this set of experiments found that a concentration of 10 μg L^{-1} read at 620nm after 2 min at 25 °C gave a linear response (Figs.3.1 and 3.2). This was followed by a rapid decline in absorbance, which suggested that the rate of reaction was very rapid. A high absorbance value is desirable in any standard assay as it is then possible to observe small differences in enzyme activity. Assay results were found to be reproducible

The method required large quantities of enzyme and was time consuming when working with large quantities of sample. A scaled-down version of the assay was therefore employed, allowing the use of a microtitre plate. The following volumes were chosen: 50 μl enzyme sample and 150 μl of buffered substrate solution.

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 the HRP present. Although TMB has an absorption peak at 650nm, no filter was available for this wavelength. The A_{620} reading was regarded as adequate.

Problems were encountered in dissolving the TMB, even at low water concentrations. It was, therefore, necessary to dissolve the TMB in DMSO (at 2% of the final buffer volume) prior to addition to the citrate buffer, pH 5.5. Extreme care was required in substrate preparation as TMB is very susceptible to photo- and thermal oxidation. All glassware used in the TMB preparation was thoroughly washed and rinsed in ultra-pure water. The TMB solution was covered in tinfoil and used immediately. Otherwise the hydrogen donor was found to spontaneously oxidise (turn blue) even in the absence of the HRP system. The concentration of H_2O_2 used in the microplate assay was 0.03% (v\v). Greater concentrations of H_2O_2 will inactivate the HRP (Gerber et al., 1985).

This microassay for HRP using the highly sensitive, non-mutagenic and non-carcinogenic TMB was used as the standard activity assay in the chemical modification studies that were carried out. Other methods of assessing the activity of HRP are available (Section 1.5)

9.1.2 ABTS Assay

Other hydrogen donors have been used to assay for HRP activity, one such hydrogen donor in common use is ABTS (Smith et al., 1990). Due the popularity of this substrate, comparative studies were undertaken with TMB. The thermal stability of native HRP in 10mM phosphate buffer pH 7.0 was determined using the TMB assay and the ABTS assay. The samples for the ABTS assay were read at A_{405} immediately after withdrawal from the heated water bath. The samples for the TMB assay were stored on ice prior to assay. It had been suggested that some refolding may have occurred during storage on ice, which could result in erroneous results using the TMB assay. However, the difference in residual activity between the methods was very small (Fig. 3.5). The difference was not considered to be significant and it was decided that a direct comparison could be made between the results of activity assays using both methods. The ABTS assay does not suffer from oxidative problems as TMB does; therefore, if a microplate assay was developed it would be a superior method to use for determining activity.

9.1.3 Effects of Varying Buffer Composition

Suggestions had also been made (Dr. A.T. Smith, Personal communication) that the buffer employed was of vital importance, i.e. that phosphate buffer may exert a chelating effect on HRP and thus reduce its residual activity. To test this, a thermoinactivation study at 60 °C was carried out on native HRP prepared in both 10mM MOPS (non-chelating) and phosphate buffer at pH 7.0. At 60 °C, the half life in MOPS buffer was found to be twice that of the native HRP in phosphate buffer. Samples in both buffers were incubated for 1h with 1mM $CaCl_2$ or 1mM EDTA. The $CaCl_2$ was found to have a slightly protective effect on HRP prepared in phosphate buffer. The presence of EDTA alone for 1h had a negligible effect. The native HRP prepared in MOPS buffer was

more susceptible to the addition of $CaCl_2$ or EDTA. The changes in the half life were more dramatic for MOPS buffer (Table 3.1). Therefore, while the use of MOPS buffer resulted in a higher half-life for the native HRP it also appeared to be more sensitive to external influences. For this reason it was decided that the slightly lower activities obtained using phosphate buffer were balanced out by its consistency. Phosphate buffer was used as the buffer of choice for HRP in the subsequent experiments, unless otherwise stated.

9.1.4 Thermal Stability of Native HRP

The thermal inactivation of HRP was carried out at 65 °C (60 °C for the comparison of the two buffer system). Initially, a thermostability study was carried out using 10 min exposures at increasing temperatures. It was observed that after 10 min at 75 ^{0}C, the percentage relative catalytic activity was practically zero. Incubation at 65 °C produced a slightly greater level of enzyme activity, with approximately 5% remaining after 10 min (Fig. 3.3). Also some residual activity remained after 20 min at 65 °C (Fig. 3.4). An enzyme loses activity at high temperatures due to unfolding of the polypeptide backbone. Inactivation is often caused by the destruction of one or two "weak points", such as hydrolysable peptide bonds, easily oxidised functional groups, etc. (Mozhaev and Martinek, 1982). Activity is lost if exposure to high temperatures is prolonged as unfolding disrupts the active site to an extent that is irreversible. Thermodynamic stability concerns the resistance of the folded protein conformation to denaturation, while long term stability measures the resistance to irreversible inactivation (i.e. persistence of biological activity). Both types can be represented by a single scheme (Mozhaev, 1993).

$$\mathbf{N} \overset{K}{\Leftrightarrow} \mathbf{U} \overset{k}{\Rightarrow} \mathbf{I}$$

Where N, U and I are the native, unfolded (denatured) and irreversibly inactivated forms of a protein, respectively. K is the equilibrium constant for the reversible N $\Leftrightarrow$ U transition, while

k is the rate constant for the irreversible U $\Rightarrow$ I reaction. The N $\Leftrightarrow$ U corresponds to thermodynamic stability while the U $\Rightarrow$ I reaction represents long term stability.

9.2 Chemical Modifications

An approach was undertaken whereby a number of the amino acids on HRP were chemically modified. As described in Section 4.1, a number of residues are unavailable for modification and others have previously been successfully modified. The lysine residues, for example, had been modified by Miland et al. (1996) using NHS esters. This modification was repeated and further characterisation studies were performed on it. It is only possible to modify about half of the twenty amino acid residues in proteins. A number of the modifying reagents employed are not specific for one amino acid residue. However, certain residues, because of their microenvironment within the three dimensional structure of the enzyme, exhibit reactivities different from those expected on the basis of studies of model systems. Often, these amino acids are the ones involved in the biological function. These may possess unusual chemical reactivities and, on this basis, they can be modified selectively (Riordan, 1979). Also, it is possible for a variety of reagents to be employed to modify the same residue. Using this approach, some residues were modified with a number of different reagents.

9.2.1 Modification of Arginine by Treatment with Diacetyl and Glyoxal

A HRP concentration of 1mg ml^{-1} was used and a 15- or 10- fold molar excess of diacetyl or glyoxal respectively was employed. There are 21 arginine residues in HRP (Welinder, 1979). All of the reactions were carried out in 10mM phosphate buffer, pH 7.0. The pH change was monitored throughout both reactions and was found to be negligible. A relatively high percentage of recovery was noted after both diacetyl and glyoxal modifications, i.e. 87% and 78% respectively. This is in contrast to Adak et al. (1996) who reported that the arginine specific reagents diacetyl and glyoxal totally inactivated HRP. Urrutigoity et al. (1993) reported that the blocking of guanidine groups destabilised the enzyme-H_2O_2 complex, by disrupting interactions between oxygen (on H_2O_2) and hydrogen

(on the guanidino N atom of Arg). Nevertheless, enzymatic activity was not lost following this modification.

It has been reported by Fliss and Viswanatha (1979) that exposure to light of arginine residues modified by diacetyl resulted in inactivation of the enzyme. This inactivation, they suggested, was independent of the degree of modification of arginine. With this in mind, both arginine modifications were carried out both in the presence and absence of light. It was found that the presence or absence of light during the modification procedure had no effect on the activity of modified enzyme (Figs 4.1 and 4.2).

While a high percentage recovery was obtained following the modification procedure, no increase in thermostability was noted (Figs 4.1 and 4.2). The modified enzymes were incubated with THF and DMF at room temperature for 1h. At low concentrations of DMF the activity was enhanced for the modified HRP and some activity was retained up to 40% DMF. No increase in organotolerance, however, was noted in the case of THF.

As no increased thermostability or organotolerance was noted in the diacetyl/glyoxal HRP no further characterisation studies were undertaken. However, arginine (particularly Arg38; Section 1.2) has been shown to play an important role in the aromatic binding site. Therefore, it is feasible that binding studies would reveal that the modifications, whilst not enhancing the thermal stability, would have a significant effect of the binding capabilities of HRP. In fact, studies carried out by Adak et al. (1996) demonstrated that HRP fractions modified with the arginine-specific reagents diacetyl and glyoxal were unable to bind guaiacol. It has been suggested that changing this charged residue to a neutral one would decrease the stability of the precursor enzyme-substrate complex and inhibit the subsequent heterolytic cleavage of the oxygen bond (Roderiguez-Lopez & Smith, 1996b).

Site directed mutagenesis of the Arg38 site has revealed similar results (Section 1.2). A marked increase in K_m for H_2O_2 was noted by Urrutigoity et al. (1993) when HRP was modified by diacetyl. (This, unlike Adak et al. (1996) was without loss of activity). Therefore, while no increase in thermostability or organotolerance was noted with this set of experiments it is likely that, had binding studies been carried out, substantial changes would

have been observed. However, at this early stage of the experiments the main aim was simply to determine modifications that increased the thermal stability and organotolerance characteristics of HRP.

9.3 Nitration of Tyrosine Residues with TNM

Chemical modification studies, as stated, often provide important information regarding the roles of the amino acid residues and the reactive mechanisms of enzymes. Such studies lead to the identification of which amino acids are contained within the active site of an enzyme. His 42, Arg38, Phe142 and Phe 143 have been implicated in the case of HRP (Veitch et al., 1995). Tyrosine residues are thought to be involved in the active site of a number of enzymes such as Carboxypeptidase (Riordan et al., 1967) and Lipases (Gu & Sih, 1992; Kawase et al.,1991). Kiss et al (1981) suggested that the tyrosine side chains of β-D-glucosidase enzyme have a substrate- binding function. Tyrosine residues have not yet been implicated as having a role in the catalytic cycle of HRP.

9.3.1 Optimisation of the TNM Reaction

The reaction with TNM was carried out over a range of molar excess of TNM. The values ranged from a 1:1 stoichiometry to 2000-fold excess. A 10-fold molar excess incubated for 30 min at room temperature resulted in the highest percentage recovery (Table 4.2). Modification with TNM produced an interesting effect on the activities. As noted in Section 4.5.2.2, while the percentage of activity recovered after incubation with 10-fold molar excess of TNM was high, there was no increase observed in its thermostability at 65 °C. Indeed, the organotolerance of TNM-HRP on exposure to DMSO, THF and DMF was no better than for the native HRP.

Studies have demonstrated a relationship between the activity of Lipase (from *Pseudomanas sp.*) and the number of tyrosine residues modified. It was found that modification of one or two tyrosine residues of the 15 in Lipase (5 in HRP) was enough to regulate enzymatic function. The esterase activity was decreased to 70% by modification of

one residue, but the activity recovered with an increase in the number of tyrosine residues modified. A possible explanation for this phenomenon is that the conformation around the active site of the enzyme was perturbed by modification of one tyrosine, but that this effect was cancelled out by further modifications (Kawase et al.,1991). Other Lipases (e.g. from *Candida cylindracea*), appear to have relatively exposed tyrosines which were all modified by 100-fold molar excess of TNM. The enantioselectivity of this Lipase was then markedly enhanced (Gu & Sih 1992). There is no evidence that the tyrosines of HRP are exposed and, therefore, easily available for modification by TNM. The fact that increasing the amount of TNM from 10-fold to 2000-fold had no significant effect on the activity suggests that all the available tyrosines have been modified at this stage. The incubation time is an important factor. The percentage activity recovered between 30 and 60 min reaction was high, i.e. 96-97%. However, any further increase in incubation time resulted in a gradual loss in activity (Table 4.2). Only 70% activity remained after 120 min incubation. Kiss et al. (1981) used incubation times of 40 min, unlike Gu & Sih (1992) who reported that a 46h incubation was necessary. Binding and kinetic studies were not carried out for this modification, as it was felt that the tyrosines do not play an important role in the catalytic cycle or in the aromatic donor binding site. This theory is supported by the findings of Adak and co-workers (1996) who modified HRP with TNM. While 50% of the activity was lost, they were able to perform binding studies. It was found that guaicol was bound to HRP with only slightly reduced affinity. Neither the ability to form Compound I with H_2O_2 nor the reduction of Compound II to native by guaicol were affected. These are further indications, that binding capabilities were not affected.

9.4 Modification using N-Hydroxysuccinimides

NHS esters are popular as protein modifying agents because of their mild and speedy reaction conditions and their high chemical reactivity and specificity. One drawback is the fact that NHS esters are not readily soluble in aqueous buffers (Ji, 1983). The NHS esters must be dissolved initially in a minimal volume of organic solvent such as DMSO or DMF. The effects of charge neutralisation through chemical modification of lysine residues and the effect of crosslinking using NHS esters are investigated.

9.4.1 EG-NHS Modification of HRP

Chemical modification of HRP's lysines with NHS esters has previously yielded derivatives with improved thermostability (Ryan et al., 1994) and this modification procedure was further optimised by Miland et al. (1996).

The EG-NHS modification was carried out at a concentration of 1mg ml^{-1} with a reaction time of 20 min. The termination of the reaction was different to the method used by Ryan et al. (1994) and Miland et al. (1996). In their previous methods, the reaction was stopped by the addition of an equal volume of Tris buffer. However, along with the problem of contamination of the sample with Tris buffer, there was also the problem of dilution of the sample. Dilution was not desirable as a number of assays (e.g. the fluorescence assays, Section 2.17.1) required a high concentration of the modified HRP. The termination was therefore carried out by Sephadex G-25 centrifugal gel filtration (Section 2.7). This method was found to give recoveries equal to those obtained using the Tris method (i.e. recoveries in excess of 95%).

The TNBS assay was carried out initially to determine the number of lysines modified. The results of this were compared with the fluorescamine assay for the determination of amino groups. Both results were in agreement with each other. It was determined that approximately 3 of the 5 available lysines were modified by EG-NHS.

Bis-N-hydroxysuccinimide esters are homobifunctional crosslinking reagents i.e. they have two reactive groups, one at either end of the molecule. The type used to crosslink HRP in these experiments is ethylene glycol-bis (succinic acid) (EG) ester of NHS and its maximum molecular linkage is 16A (See Fig. 4.5). The size of the crosslink used can have a drastic effect with some enzymes, e.g. modification with SA-NHS decreased the activity of trypsin to 38% while EG-NHS increased the activity to 203% (Murphy ph.D. Thesis, 1996). Torchilin et al (1983) found that the length of the carbon chain in the intramolecular crosslink influenced the degree of thermostability of alpha-chmyotrypsin.

Modification of the lysine residue of HRP by EG-NHS is accompanied by a slight loss in activity, i.e. 95% recovery. EG-HRP demonstrated increased resistance to elevated temperatures. At 65 °C the half-life of EG-HRP was 10 times greater than that of

the native HRP. From the temperature profile (Fig. 4.3) the T_{30} value for EG-HRP was approximately 50 °C higher than native (55 °C and 5 °C respectively). Mozhaev (1993) outlines a two step model of irreversible thermoinactivation that involves a preliminary reversible unfolding and an irreversible step (Section 9.1.4). The hydrophobic probe Nile Red is used to try to elucidate the degree of unfolding as a result of heat treatment of the native and modified forms. This is discussed in detail in Section 9.8.

Chemical crosslinking of the protein is generally thought to stabilise it against reversible unfolding. The fact that 3 of the 5 available lysines have been modified implies that at least one, and possibly two, molecular crosslinks have been formed on the polypeptide backbone. Neutralisation of the positive charge of the lysine amino group has a stabilising effect even in the absence of crosslinking. (This is demonstrated with the phthalic anhydride modification, Section 9.5).

Tolerance of organic solvents (DMF, THF, DMSO and 1,4-dioxane) was investigated. In most cases the native HRP was inactivated as the percentage of organic solvent increased. But, the EG-HRP derivatives were found to be more organotolerant than the native. The modification of HRP (by the formation of crosslinks) brings about significant improvement in peroxidase stability in DMF. Native and modified forms appear to be more stable in DMF than in 1,4-dioxane or THF.

9.5 Modification with Aromatic Anhydrides

A large number of acylating agents have been used for modification of ε-amino lysine residues in proteins (Bagree et al., 1980). A good modifying agent should be specific for a group under certain conditions. Certain anhydrides have been found to conform to this condition (Mozhaev et al., 1988). Covalent modification of protein surfaces with a strongly hydrophilic low-molecular-weight group could conceivably help the protein to hold its hydration 'shell' tightly and in that way, resist the denaturing effect of the stripping of water from its surface by organic solvents.

For the acylation of amino groups of HRP, a series of aromatic carboxylic acid anhydrides was used. As a result of these modifications, several new carboxylic groups are introduced into the HRP. The anhydrides used were phthalic, trimellitic and pyromellitic

anhydrides. As a result, one, two or three negatively-charged carboxylic groups were introduced into each Lys residue modified. Pyromellitic anhydride is a bi-functional reagent and intra- and inter-molecular crosslinking may, in principle, proceed simultaneously with mono-functional modification of the enzyme. However, experiments by Mozhaev et al. (1988) have eliminated this possibility. It does, however, remain one of the most popular anhydrides for covalent modification of enzymes, particularly α-chymotrypsin (Khmelnitsky et al.,1991).

A HRP concentration of 1mg ml^{-1} was used with a 100-fold molar excess of each of the anhydrides over available NH_2 groups. The reaction mixtures were incubated at 4 °C for 2.5h while stirring continuously. The excess reagent was then removed according to the method developed for EG-HRP, i.e. Sephadex G-25 gel filtration (Section 2.7). The percentage of activity retained after the modifications was high. There was in excess of 90% for phthalic anhydride and 80% for both trimellitic and pyromellitic anhydride (Table 4.1).

The thermoinactivation of the native and modified forms of HRP is shown in Fig. 4.7. All the modified forms with the exception of phthalic anhydride (PA) showed a very slight enhanced thermostability compared with the native. In the case of phthalic anhydride, there was a 5-fold increase in thermostability at 65 °C compared with the native. Since the PA HRP demonstrated most stability at elevated temperatures, it appears that the addition of one negatively-charged COOH group onto the Lys residue is the most advantageous with regard to both the percentage of activity recovered after the modification and the enhanced stability. A number of the Lys residues of HRP are contained within regions of varying hydrophobicity (Table 9.1). Neutralising the charge on at least two of these improves both the thermal stability and the organotolerance. It was determined that 2 of the 5 available lysines are modified with phthalic anhydride. Any further increase in negative charge results in only a marginal increase in stability.

As the pyromellitic and trimellitic anhydride-modified HRP demonstrated very little enhanced thermostability, they were not investigated further. There is a 5-fold increase in the thermostability of the PA HRP compared with the native at 65 °C. This is less than the 11-fold stabilisation noted with the EG HRP. It would appear, therefore, that the increased stability with EG HRP arises from the increased structural rigidity of the enzyme

as a result of crosslinking, which prevents the unfolding of the molecule. Both EG-and PA - HRP retained the same percentage of activity at 55 °C (approximately 88-90%) (Fig. 4.4 and Fig. 4.8). After 10 min at 70 °C ≈20% and ≈40% activity remains for the PA HRP and EG HRP respectively, compared with values of almost zero for the native. Therefore, at the higher temperatures the crosslinking modification affords better stabilisation to the enzyme. While the degree of thermostability attained by the PA HRP is not as dramatic as for the EG HRP, it is nevertheless a substantial effect.

Tolerance of organic solvents (DMF, THF, methanol and 1,4-dioxane) was enhanced with PA HRP. Methanol, DMF and THF possess respectively low, medium and high denaturation capacities, as defined by Khmelnitsky et al. (1991). The native and modified HRPs were inactivated more rapidly in the presence of THF than in DMF. The acylated form of HRP was able to withstand the effects of DMF as well as did EG HRP. The combined effect of elevated temperature and organic solvent was investigated. (Fig. 4.12). At 65 °C, activities for the native HRP were practically zero at 20% THF. However, the PA HRP was more tolerant of the combined effects, with over 20% activity remaining at 20% THF. Extreme care had to be exercised when analysing the THF samples. The plastic wells of the microplates were prone to "frosting" at high % (v\v) THF. It was, therefore, necessary to read the absorbance values immediately after transfer to the plates. Concentrations above 50% THF could not be determined even with these precautions.

As a result of the PA modification, a negatively charged group is introduced in HRP. The result is an increase in hydrophilicity of the HRP molecule (Mozhaev et al., 1988). As has been demonstrated the hydrophilised HRP was able to endure much higher concentrations of organic solvent with loss of catalytic activity compared with the native. These results indicate that hydrophilisation brings about a significant increase in protein stability against denaturation by organic solvents. The explanation is that the increased hydrophilisation of the enzyme enhanced its ability to retain its hydration water.

The positions of the lysine residues and their availability for modification are essential to the understanding of the above systems of modification. Comparison of the hydropathic character (positive hydropathy indicates hydrophobicity) of the amino acid sequence of HRP C (and CCP) is outlined by Welinder (1985). The location of the lysine

residues was determined from the amino acid sequence for HRP C published by Welinder (1979). The stereoscopic views of the backbone of CCP show a close agreement between "hydrophilicity and exposure" and "hydrophobicity and interior location". Exceptions might occur where large hydrophilic side chains of a buried backbone residue reach the protein surface, or deep-lying catalytic residues in the active site give rise to some internal hydrophilicity.

Table 9.1 **Position of Hydrophobic Regions in HRP C**

Residue no.[a]	No. of Lys	Position [a]	Degree of hydrophobicity [b]	Helices and Segments
40 -50	-----	-----	+++	B
55 -70	1	65	+++	β1
80 -85	1	84	neg	gap
87 -100	-----	-----	+	C
110 -125	-----	-----	+++	D
140 -160	1	149	++	gap
172 -182	1	174	+++	F
229 -236	1	232	+++	gap
237 -245	1	241	+	β3

[a] from amino acid sequence of HRP C (Welinder, 1979),
[b] from hydropathic character map (Welinder, 1985),
+ indicates the degree of hydrophobicity.

The structural model predicts that the B, D and the end of the F helices are the most buried. One lysine (Lys174) is within the domain of the F helix which, from Fig 9.1 can be seen to be located near the haem. This supports the assumption that one of the lysines is buried deep within a hydrophobic environment and is partially shielded by the haem, thus, making it unavailable for modification. There are 2 lysines (Lys84 and Lys241 (β3)) contained within the regions of low hydrophobicity.(Fig. 9.2). These could conceivably be available for covalent attachment with phthalic anhydride. Alternatively, the lysines in the hydrophobic regions could be protruding from the area. These are Lys65 (β1), Lys149 (gap) and Lys232 (gap). As lysine is quite a long chain, it is possible that the positive R-group at the end is far enough away from the hydrophobic region that the attachment of the anhydride does not interfere with it. Also the hydrophobic benzene ring of the PA will not disrupt a hydrophobic patch.

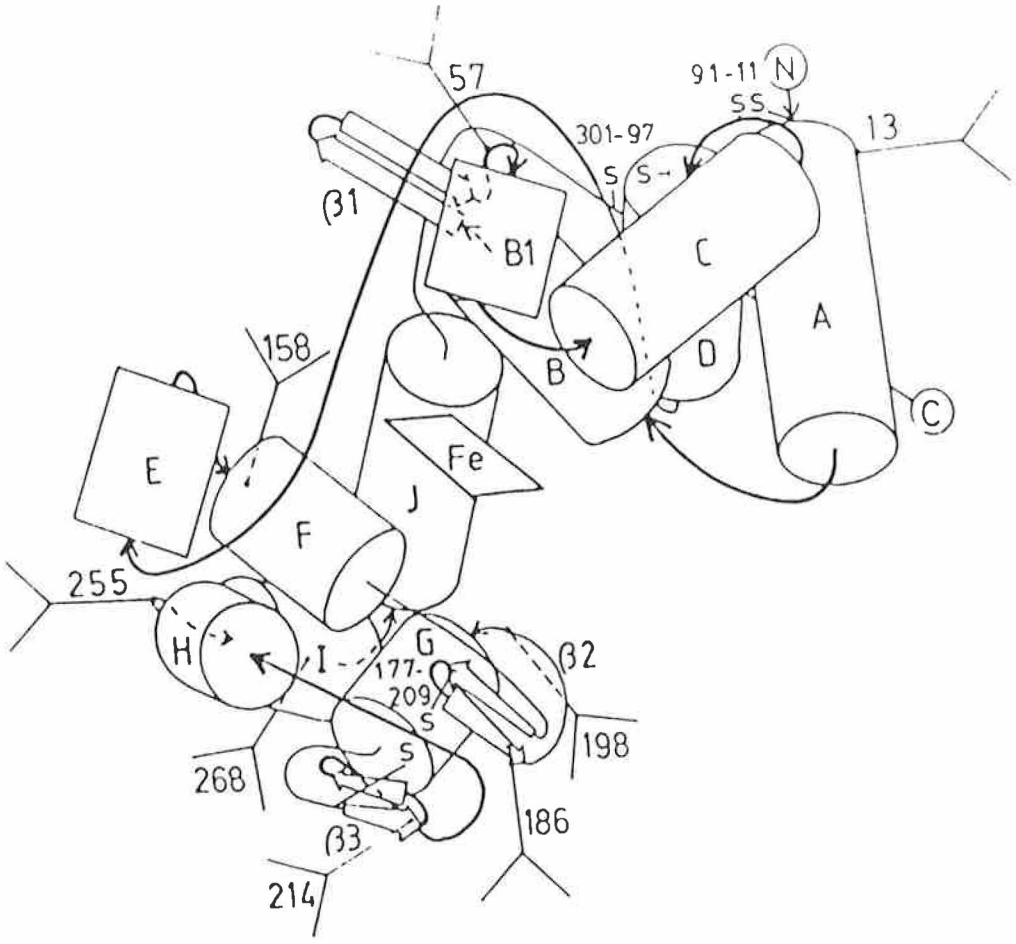


Fig. 9.2 Structural model of peroxidase. The model is constructed from a store picture of cytochrome c peroxidase, (Welinder, 1985)

9.6 Hydrogen Peroxide Stability

In the absence of a reductant substrate and with excess hydrogen peroxide, native peroxidase yields firstly the active intermediate enzymatic form called Compound I. An excess of hydrogen peroxide results in enzyme inactivation, the reaction between Compound II and hydrogen peroxide results in the formation of Compound III (Scheme 1.2). In this study the inactivation process taking place in the reaction between peroxidase and excess hydrogen peroxide in the absence of a reductant substrate is studied, for native and modified HRP.

A typical visible spectrum of native peroxidase is shown in Fig. 4.13, trace A. When H_2O_2 is added, the spectrum changes; there is a red shift which is followed by a reduction in the absorbance of the Soret band with increasing time of exposure to peroxide. From these scans the half life of native HRP and its derivatives at 25 °C over time could be determined. Arnao et al.,(1990) carried out a kinetic study on the suicide inactivation of HRP by hydrogen peroxide. They were able to demonstrate a protective role for Compound III against hydrogen peroxide-dependent peroxidase inactivation.

The k_1 value (reduction of H_2O_2) was not determined in this set of experiments. It was decided to determine the half life of HRP and its derivatives on incubation with peroxide over time at 25 °C. Any increased resistance to peroxide could be beneficial in some of the environmental applications of HRP. The decrease in absorbance at 405nm was monitored. The differences in the $t_{1/2}$ for the native and EG-HRP were found to be negligible, 39 and 38min respectively. However a small decrease in the half life of PA HRP was noted (33 min). This slightly reduced ability to withstand peroxide attack does not appear to have any significant effect on any of the functions or catalytic activity of PA HRP.

9.7 Enzyme Immobilisation

Samples (1mg ml^{-1} concentration) of native, EG-, and PA-HRP were immobilised on adipic acid dihydrazide-agarose (AADH-A). Also, 100μg L^{-1} of native, EG-, and PA-HRP was immobilised in calcium alginate beads. 70% of the AHA NHS HRP was recovered after modification. It was necessary to dilute this immobilised form in 10mM

phosphate buffer, pH 7.0, prior to assay. It was found that 1:1000 dilution for native and 1:100 for both modified forms gave absorbance values that corresponded to a concentration of 5μg L^{-1} when compared with soluble HRP assayed under similar conditions.

Alginic acid is a constituent produced by the brown algae of Phaeophyceae which occur in intertidal zones in, for example, the Sargasso sea. There are many commercial sources, principally *Macrocytis pyrifera,* the giant kelp which is harvested off the Pacific coast (Bucke, 1987).

The production of the alginate beads and entrapment of HRP was very successful. The beads were easy to handle and did not disintegrate on drying. Also, it was relatively easy to store the beads in sealed containers. (It was important, however, to keep them moist to prevent irreversible shrinkage).

A number of problems were encountered with the beads, such as enzyme leakage. On addition of hydrogen peroxide to the system, the leakage could clearly be observed as the emergence of a blue stream of oxidised TMB from the beads. For this reason, no further tests were carried out on this immobilised form. Problems have also been encountered by other workers using calcium alginate beads for entrapment. They found that (1) the bead is rapidly disrupted by chemicals capable of chelating calcium ions and (2) the beads tended to swell in the pressure of monovalent systems (Bucke, 1987).

One potential use of this system for enzymes is the long term storage of modified forms. The moist beads stored in a sealed container for 6 months were assayed for activity. 70% residual activity remained after this time period. The immobilised enzyme was useful for only a single assay.

The thermostabilities at 65 °C of the AADH-A forms of the native, EG- and PA-HRP were determined. The half lives of the modified forms were almost 3-fold greater than the immobilised native. The $t_{1/2}$ values for the native, EG- and PA HRP were 9 min, 22min and 26min respectively (Fig. 4.15). These half lives were compared with the $t_{1/2}$ values for their soluble counterparts at 65 °C. It was noted that the immobilised native and PA HRP were 3.5 and 2 fold more stable than soluble native and PA HRP at 65 °C. However, immobilised EG HRP, while more thermostable than the native was no more stable than soluble EG HRP. This may be due to the nature of the modifications. The EG, as described

earlier, is a crosslinking reagent, while PA is a covalent attachment. Studies have shown that binding the enzyme to a solid support results in a more rigid structure of the protein (Klibanov, 1982). That is, the multipoint attachment to a solid support (without necessarily altering the enzyme) makes it more stable against unfolding and ultimately more resistant to thermal inactivation. Therefore, the more points of attachment there are, the more resistant to unfolding the immobilised enzyme will be (See Fig. 9.2). It may be that due to the nature of the modifications, the PA HRP has more easily attached points for immobilisation than the EG HRP. The AADH-A gel presents a dihydrazide group which can react with the carboxylic groups of the enzyme's Glu and Asp residues(Moreno et al. 1996). The PA modification, as mentioned, adds an extra carboxylic group on to the lysine residues. This possibly favours immobilisation on the AADH-A gel.

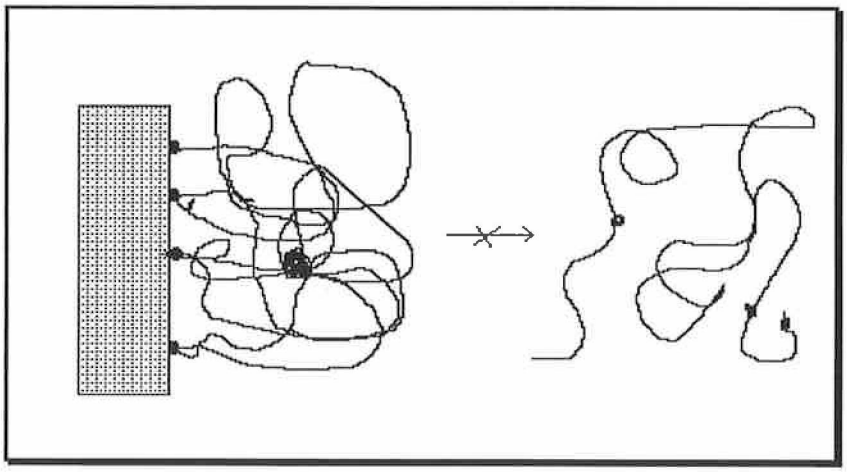


Fig. 9.2 Schematic representation of how covalent attachment to a solid support by many linkages stabilises the enzyme against thermal unfolding.

9.8 Unfolding HRP

The effects of denaturants and reducing agents on native and modified HRP were determined. Urea is a frequently used denaturant; however, urea usually has little effect

effect on HRP even at concentrations as high as 8 M. After 1h incubation with 8 M urea at room temperature, the native and modified HRPs (EG and PA) still maintained up to 94% activity. The inclusion of 1mM EDTA for a further 2h had only a minimal effect, reducing the activity to 75%.

On incubation with guanidine chloride (GnCl) it was found that 4.5M GnCl was required to reduce the activity of the native by 50%. This value was increased to 6M GnCl for the modified forms of HRP. Pappa and Cass (1993) observed that exposure to 0-1M GnCl induces no conformational change in the protein, while exposure to 1-5M GnCl unfolds the backbone (this was shown by CD, tryptophan fluorescence and haem intensity); however, the haem remained bound to the protein. At concentrations higher than 5M GnCl, the protein is completely denatured and the haem falls out of the protein.

The importance of calcium in maintaining the structural stability of HRP has been demonstrated by Haschke and Friedhoff (1978) and also by Smith et al (1992). It was reported that while many proteins contain calcium as a component, HRP appears to be the only one where it is an essential constituent. Correct refolding of HRP will not take place in the absence of calcium. To investigate this, both native and modified HRP were incubated with EDTA and GnCl. Urea could not replace GnCl as a denaturant and EDTA alone removed the calcium only very slowly. (The extent of calcium removal was determined by HRP activity after the incubation period). A considerable difference was noted between the native and modified HRP. The C_{50} values (the concentration at which 50% of the HRP activity remained) for the native, EG and PA HRP were 1.1mM, 2mM and 3mM respectively (Table 5.1). The calcium free enzyme is considerably less stable than the untreated forms.

The reducing agent TCEP gave similar results, with the modified forms demonstrating more than 2 fold increased resistance to the reducing effects of TCEP. TCEP reduces the disulphide bridges of HRP, which have been shown to be important in the overall molecular stability of the enzyme (Pappa and Cass, 1993).

The use of the hydrophobic probe Nile Red confirmed the unfolding in the native HRP. It also showed that a certain degree of unfolding at high temperatures was still occurring in the modified forms. It is possible that the modification offers some protection

against incorrect refolding rather than completely preventing unfolding of the structure. Fig. 5.4-5.7 show that the unfolding has been significantly reduced in EG HRP, however, the difference with the PA HRP is not as apparent. The heat treatment resulted in a progressive change in the enzyme which caused a characteristic blue shift in Nile Red emission from 630nm to about 610nm and enhanced the fluorescence by as much as 7-fold. Denaturation by GnCl would destroy the spectral shift. The Nile Red would then have the same fluorescence characteristics in the presence or absence of denatured protein (Sackett and Wolff, 1987).

These results were compared with unfolding studies carried out at 65 °C using tryptophan fluorescence to determine the extent of unfolding. There is one tryptophan residue in HRP buried deep in the interior of the molecule. This can, however, become exposed as a result of unfolding and its fluorescence intensity determined. The native and modified HRP were each placed in a heated cuvette linked to the fluorimeter and the fluorescence emission scanned continuously over 2h. This differed from the Nile Red method (where the samples were plunged into ice for 30sec after heating and prior to measuring the fluorescence). While the methodologies differed, the results obtained agreed. Both methods indicate a higher degree of unfolding for the native than the PA or EG HRPs. It would appear, then, that the temperatures used for the Nile Red experiments were high enough to induce irreversible unfolding.

The activity after thermoinactivation at 65 °C is shown in Fig. 5.11 compared with the unfolding. The percentage of activity lost can be compared directly with the extent of unfolding noted at the elevated temperatures. Husain et al. (1992) stated that immobilised HRP could recover up to 60% of its activity after thermal inactivation at 70 °C, by incubation at 0 °C. The extent of activity recovered was inversely proportional to the length of exposure to 70 °C (and, presumably, the temperature). Reactivation of heat-denatured enzymes is better accomplished if the polypeptide is first completely unfolded prior to exposing it to conditions that facilitate refolding, (Klibanov & Mozhaev 1978). No definite refolding experiments were undertaken, except for the observations of the Nile Red and tryptophan fluorescence. The refolding experiments are hard to perform and usually result in low activity (Pappa and Cass, 1993; Smith et al. 1992).

9.9 Digestion of Holo/Apo Enzyme

There are, as stated previously, 6 lysine residues in HRP, with only 5 available for modification. While it is possible to determine the number of lysines modified by either of the two modifications it has not been possible to identify which lysines were modified. To this end, chemical and enzymatic digestions were undertaken. Cyanogen bromide (CNBr) reacts with methionine residues and has been used for the non-enzymatic cleavage of the methionyl peptide bond (Gross &Witkop 1967). There are four methionines in HRP at positions 83, 181, 281 and 284 (Welinder, 1979). CNBr should cleave the apo enzyme at each of these points, resulting in five fragments. Therefore, depending on the position of the lysine crosslinks there should be less fragments in the modified HRP. From the molecular weights of these fragments and different RF values i.e. if two fragments are joined by EG modification, it should be possible to determine the position of the lysines modified. The trypsin and Endoproteinase Lys C provide similar information. They are specific for lysine/arginine and for lysine respectively. These digestions would, therefore, result in the formation of at least seven fragments in the native HRP. Comparison of the fragment bands in the modified HRP with the different methods could result in building a "map" of the positions of the modified lysines.

The digested samples were visualised by SDS-PAGE electrophoresis. A number of different percentage gels and different gel systems were tried. This included the Anderson discontinuous urea-SDS PAGE, which has been used successfully to study both native small polypeptides and CNBr polypeptide digests. (Hames & Rickwood 1990). The Anderson method proved difficult to use and the results were no better than with the Laemmli (1970) system. Therefore, the latter method was employed.

The methods of enzymatic digestion described above were carried out exhaustively over a number of months, the results, however, still proved inconclusive. Firstly, the commercial HRP was not pure and the native control showed up as multiple bands on the gel instead of a single band at 44,000Da. While incubation with the CNBr succeeded in fragmenting the native apo HRP, the bands were not present in the numbers or size expected. There was an absence of any band except for a faint band at 44,000Da for

both of the modified forms. Welinder (1979) found that for the native HRP, CNBr hydrolysis was incomplete, even using 50 times molar excess. This, she hypothesised, was due to the high carbohydrate content of HRP. The trypsin and Endo Lys C digestions yielded similar results. There was, however, a band at less than 14,000Da present with the Endo Lys C digestion that was not apparent for the CNBr digestion (Figs. 5.12a and 5.12b).

The CNBr fragments were analysed by HPLC. The profile of the samples obtained using this method was no more informative than the results using the SDS-gels. An example of the profiles obtained is shown in Fig. 5.13. As the recombinant HRP does not contain a carbohydrate moiety, then it should be possible to successfully perform the CNBr digestions on HRP C*.

9.10 Recombinant HRP (HRP C*)

The carbohydrate portion of a glycoprotein can greatly stabilise the polypeptide against denaturation and/or inactivation. Glycosylated erythropoietin, for instance, is more resistant to high temperatures and guanidine hydrochloride than the 'naked' polypeptide (Narhi et al.,1991). Similarly, Bonnaffe et al. (1993) have reported that chemical attachment of additional carbohydrate can stabilise HRP.

The results described here suggest that *E. coli*-expressed HRP C* of Smith et al. (1990) is 4 - 5-fold more stable than the plant enzyme at 65 °C (Table 6.1). This contrasts markedly with Egorov et al. (1994) who reported Chiswell and Ortlepp's (1987) *E. coli*-expressed, unglycosylated, recombinant HRP to be 3-fold *less* stable at 56 °C than the plant enzyme. Bonnaffe et al. (1993) found that periodate-deglycosylated plant HRP was 2.8 times less stable at 50 ° C than untreated enzyme, while Hiner et al. (1995) noted that carbohydrate-free HRP C* was twice as sensitive as plant HRP to H_2O_2 inactivation.

The present experiments and those of Egorov et al. (1994) used different HRP sources (both plant and recombinant) and inactivation temperatures of 65 ° C and 56 ° C respectively. [A comparative 65 °C inactivation (Section 3.2.5) has revealed stability differences between various commercial sources of plant HRP]. Here, heat exposed samples were held on ice prior to simultaneous assay of all samples, while Egorov et al. (1994) assayed each inactivation sample immediately following removal. However, thermoinactivation studies carried out using ABTS

(assaying immediately) and TMB (storing on ice until the end of the allotted time period) assays, revealed no significant differences between the two methods (see Fig 3.2.4). Proteins inactivate according to the model N $\leftrightarrow$ U $\rightarrow$ I. N is the native (and only active) form, U is reversibly unfolded and I is irreversibly inactivated (O Fagain, 1995). If Ca^{2+} from unfolded HRP molecules was not completely complexed by phosphate buffer, some degree of refolding may have occurred during ice storage, so that the residual activities may represent (N + refolded U) and not N alone. However, as stated, no difference was observed between data obtained from immediate or delayed assay of time samples in the control thermoinactivation of plant HRP. Neither was there any difference between the plant HRP samples thermoinactivated in phosphate buffer in the presence or absence of 1mM EDTA, although 1mM Ca^{2+} did have a slight protecting effect (Table 3.1). Thus, refolding (at least of plant HRP) is unlikely and the observed activities are probably due to N alone. It remains possible, however, that carbohydrate-free HRP C* may have been better able to refold during storage prior to assay.

It is clear from the full recovery of initial activity that the 'naked' HRP C* polypeptide tolerates chemical modification of its lysines with EG-NHS, indicating that glycosylation does not influence HRP's ability to withstand the modification reaction without inactivation. Modification with EG-NHS stabilises HRP C*, just as it does the plant enzyme (Ryan et al., 1994): EG-HRP C* tolerated heat (Table 6.1, Fig 6.2) and 1,4-dioxan (Fig. 6.3) significantly better than did unmodified HRP C*. The stabilisation factor of 5-fold at 65 °C is, however, lower than the 23-fold stabilisation reported by Ryan et al. (1994) for plant HRP at 72.5 °C. This difference may be due to the different thermoinactivation temperatures. While EG-HRP C* showed improved tolerance of 1,4-dioxan, it was no more tolerant of DMF than was the untreated HRP C*. This contrasts with Miland et al.'s (1996) report of improved DMF tolerance following acetylation of plant HRP. Glycosylation may influence HRP's tolerance of certain solvents while having less effect on its thermal stability. Unlike the chemical modifications carried out on the plant HRP (see Section 4.0) it was not possible to quantitate the extent of lysine modification with trinitrobenzsulphonic acid (TNBS), due to the limiting quantities of HRP C* available. It is possible that the present modifications were not as extensive as those reported previously by Ryan et al.(1994). Nevertheless, it is clear that the chemical modification of HRP C* lysines significantly increases the recombinant enzyme's stability, either by preventing

unfolding (of N to U) or by facilitating refolding (of U to N). This is likely due to crosslinking, since EG-NHS is a bifunctional reagent. Stabilisation may also arise from neutralisation of lysine positive charges by EG-NHS. Three of HRP's six lysines lie in hydrophobic regions (Welinder,1985).

The use of chemical modification in modulating the properties of various proteins has been reviewed by Smith et al. (1993). Chemical modification can usefully complement a mutagenesis-based protein engineering strategy (O Fagain, 1995). It has been shown here that the carbohydrate-free, recombinant HRP C* can withstand EG-NHS modification with excellent recovery of activity and that the resulting derivative is stabilised against heat and against 1,4-dioxan. These findings re-emphasise the importance of lysine residues to HRP's stability. They also indicate that the carbohydrate moiety of HRP is not as essential to stability as was first thought.

9.11 Kinetic Studies of Native and Modified HRP

9.11.1 Characterisation Studies

Under steady-state conditions with ABTS as substrate, EG HRP exhibited a lower turnover number (83%) (Table 7.2) than native HRP, while PA HRP was more active (133%). The values of k_3 measured for the native and modified forms were similar to the values obtained for HRP C1a (variant) (Dr. A.T. Smith, personal communication 1995) see table 7.11. The k_3 values were obtained for a number of substrates using the pre-steady state method. There was, however, a problem with some of the results obtained (Table 7.4) and the k_3 value for ferrocyanide was therefore worked out again using steady state methods (Table 7.3). The k_3 values obtained for the native and modified forms in this way were compared to FPLC purified samples. As Table 7.3 shows there was very little difference between the purified and unpurified samples.

Direct determination of k_3^{ABTS} by pre-steady state methods is not possible owing to the absorption of the cation radical product in the Soret region (Smith et al., 1993), The most obvious difference between the parameters for the native, EG and PA HRP in Table 7.2 is the

higher apparent value for K_m^{ABTS} of the latter two. It could be that, due to the modifications, access of the bulky charged substrate to its binding site is somewhat more restricted than in the native. The faster turnover of PA HRP may be attributed to a faster rate of product dissociation. This apparent change in substrate specificity with respect to ABTS, ferrocyanide, ferrulic acid and indole 3-propionic acid (IPA), is possibly associated with a slight perturbation of the substrate binding site. As discussed in Section 1.2.8 alterations in or near the binding site by chemical modification or site directed mutagenesis can have profound effects on HRPs ability to bind alternative substrates such as benzhydroxamic acid (BHA). The binding site is believed to lie 8-12Å from the haem iron (Sakurada et al, 1986).

To investigate this point further, the dissociation constant K_d for the binding of BHA was determined. BHA binds with high affinity and perturbs the spin and ligation state of the haem iron from the predominantly (80%) high spin pentacoordinate system (hs5c) to a high spin hexacoordinate (hs6c) (Smith & Loew 1992). EG and PA HRP showed slightly less affinity for BHA than the native; of the two forms PA HRP had the lesser affinity. This may suggest that there have been changes to the reducing substrate binding site as a result of the modifications. Alternatively, PA modification may in some way interfere with either Arg38 or His42 (which have been implicated as important in the binding site Section 1.2.11) and in this way cause the reduction in the BHA binding affinity observed.

9.12 HRP Catalysed Removal of Phenol

9.12.1 Removal at 37 °C

The removal of phenolic compounds by HRP does not take place with either HRP or H_2O_2 alone. It is not a function of peroxide concentration. Carmichael et al (1985), showed, however, that multiple additions of co-substrate (peroxide) resulted in stepwise substrate oxidation, an indication of the relative stability of their peroxidase (Chloroperoxidase) over the course of the experiment. This observation is totally dependent on the concentration of the individual peroxide spikes, as the presence of excess peroxide would result in Compound II formation (See Fig. 1.2), which can then be oxidised to Compound III. There is a slow removal phase after the initial removal. This may in fact be accounted for by Compound III formation, as any accumulation of HRP in this state results in loss of catalytic efficiency, (Nicell et al., 1993).

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 Ei refers to Compound I.

$$E + H_2O_2 \longrightarrow Ei + H_2O$$

$$Ei + AH_2 \longrightarrow Eii + AH^{\bullet}$$

$$Eii + AH_2 \longrightarrow E + AH^{\bullet} + H_2O$$

$$Eii + H_2O_2 \longrightarrow Eiii + H_2O$$

$$Eiii \longrightarrow E + O_2^{-}$$

Nicell (1994) has postulated that the enzyme is distributed between Compound I, Compound II and the native enzyme forms, immediately following the start of the reaction. Model predictions indicated that the maximum levels of Compound I and Compound II concentrations are achieved within the first 2 milliseconds under given experimental conditions (1mM phenol, 1mM H_2O_2, 0.64U ml^{-1} at 25 °C and pH 7.0). It was assumed that the amount of enzyme inactivation at any time is directly proportional to the quantity of aromatic substrate removed from solution.

Based on the information above and the results of conditions previously optimised for native HRP, phenol, 4-chlorophenol (4-CP) and a 1:1 mixture of the two compounds were incubated with the HRP/H_2O_2 system, as described in Section 2.28. The aim of these sets of experiments was not to optimise the conditions for complete removal but, to compare the removal efficiencies of the native and modified HRP with different phenolic compounds under standard conditions. For the incubation temperature of 37 °C, removal efficiencies were very high. As much as 95% removal was noted for HRP and its derivatives with 4-CP. Removals in excess of 70% and 80% for the mixture and phenol respectively were achieved (Table 8.1). The reaction time used was 20 min, as any further removal after this time was slow. At 37 °C, the modified HRPs proved to be as efficient at precipitating the phenolic

compounds as the native. Also, there was little difference between their relative removal capacities. The most readily removed of the compounds tested was 4-CP. Xu et al. (1995) found that by varying the structure of phenols and aromatic amines, enzymatic polymerisation of over 20 kinds of phenols and aromatic amines could be performed using HRP. They found that substrates with electron-withdrawing groups were hardly polymerised, while substrates with electron-donating groups were easily polymerised. The reaction rate was higher for m- or p-substituted substrates than for o-substituted substrates.

9.12.2 Removal at 70 °C

The effect of elevated temperature on the removal efficiencies of the compounds was studied. The conditions used were the same as for the low temperature studies. It has been shown previously that inactivation of HRP is a function of time at temperatures between 5-85 °C. A reduced removal of phenol is seen at elevated temperatures. Klibanov et al. (1983) noted that there may be a number of reasons for this. They stated that neither phenol nor peroxidase alone inactivates the peroxidative reaction. They suggested the possibility of interactions between enzymatically-generated phenoxy radicals and the enzyme's active centre. Alternatively, inactivation may be due to the adsorption of protein molecules onto polyaromatic products (Nakamoto & Machidia 1992). Also, activity is lost if HRP is exposed to high temperatures for prolonged periods, as unfolding disrupts the active site to an irreversible extent (Section 5.0).

The percentage of phenol removed at 70 °C was considerably less than at the lower temperature. As much as a 50% reduction in removal was noted. However, the modified HRP were much more efficient than the native at the higher temperature. The PA HRP, while not as thermostable as the EG HRP, was in fact a better catalyst for the removal of all three phenolic compounds from the water (Table 8.2). The stabilisation of HRP due to the EG modification is as a result of crosslinking. The PA modification on the other hand is probably due to charge reversal. It is possible, therefore, that the absence of crosslinks resulted in the uninterrupted movement of the aromatic molecules to and from the enzyme active centre. This difference may be more apparent at elevated temperatures; unfolding of the enzyme, while not enough to inactivate the modified HRPs, may be enough to allow easier access to the active site. As PA

HRP is known to unfold slightly more than EG HRP, it may be that this is, in fact, beneficial where access to the active site for the aromatic molecule is concerned.

9.12.3 Bleaching of Dyes

The use of peroxidase together with hydrogen peroxide has been suggested in bleaching of pulp for paper production, in treatment of waste water from pulp production, for improved bleaching in laundry detergents and for dye transfer inhibition during laundering (Schneider, 1994). The use of bleaching agents in washing procedures is a well-established practice. They are usually incorporated into (or sold as constituents) of a major part of the commercially available detergent compositions. Conventional bleaching agents incorporated into detergent compositions are compounds which act as precursors of hydrogen peroxide in the course of the washing procedure. Perborates and percarborates are the most common examples. The detailed mechanism of bleaching by means of these bleaching agents is not known at present. One drawback of these common bleaching agents is that they are not very efficient at the temperatures at which coloured fabrics are usually washed. Their efficiency may be enhanced by the use of accelerators. Hydrogen peroxide has been found to enhance their bleaching capacity, (Welinder, 1993).This worker also found that peroxidases could act as bleaching agents, although the mechanism has not been fully elucidated. It is believed that the enzyme acts by reducing H_2O_2 and oxidising the coloured substance (Donor substrate). She found that a number of peroxidase variants of *Coprinus cinereus* (CiP) produced in *E. coli* had better stability towards hydrogen peroxide than the wild type CiP. They suggested that smaller quantities of this peroxidase could then be used with satisfactory results.

9.12.3.1 Optimisation of the Assay

The bleaching effect of different concentrations of HRP on Chicago Sky Blue (CSB) in the presence of hydrogen peroxide and 4-hydroxy benzsulfonic acid as accelerator, was determined over time. Fig. 8.4 shows that 45nM HRP for 10 min removed the most colour (in excess of 80%). As can be seen, the bleaching effect is very fast for this dye and there is very little difference between 5 and 10 min incubation.

9.12.3.2 Bleaching at 37 °C

Using the above information and following the method of Welinder, (1993), the bleaching effects (i.e. the % of colour removed) of the native and modified HRPs, were determined. The main aim of this experiment was not to develop optimum conditions for bleaching of each dye and accelerator, but to standardise conditions for the comparison of HRP and its derivatives. In some cases, it was even possible to obtain an approximate comparison of bleaching capacities by visual observation of the reaction mixtures prior to spectroscopic determination. The % of colour removed for the four dyes in the presence of the four accelerators at 37 °C was determined. From this set of experiments it was possible to determine which (if any) of the accelerators enhanced the bleaching effect and also the degree of colour removal achieved by each of the HRPs. In general, the % of colour removed from the blue dyes was the most successful, with as much as 84% of the colour removed from Chicago Sky Blue after 10 min. (Table 8.3). At 37 °C Vanillin proved to be the best accelerator. The bleaching of Acid Red and Methyl Orange were considerably enhanced in the presence of vanillin compared with the other accelerators (Table 8.3). The modified HRPs were as good at bleaching as the native HRP at this temperature, they were, however, not noticeably better.

9.12.3.3 Bleaching at 65 °C

As vanillin and 7-hydroxcoumarin proved to be the two most efficient accelerators, they were chosen in the elevated temperature studies. A strange effect was noted when the bleaching experiments were carried out at high temperatures. Firstly the 7-hydroxycoumarin was now by far the better accelerator (Table 8.4). Secondly, while the bleaching effect with vanillin at 65 °C was still relatively good, it was, as expected less than at 37 °C. The modified HRPs were more efficient at the higher temperature than the native. This was likely due to their ability to withstand unfolding better at elevated temperatures than native HRP. Welinder, (1993) noted that the CiP variants with improved stability towards H_2O_2 were better agents. From the H_2O_2 studies carried out for the native and modified HRP, it was seen that the EG HRP had the same half life as native, with PA HRP demonstrating only marginally less sensitivity. This could possibly explain why there was little difference in the bleaching capacities of the three forms at 37 °C and only a small difference at 65 °C. This latter difference was

probably due to the increased thermostability of the chemical derivatives and not due to any increased tolerance toward hydrogen peroxide.

9.12.3.4 Bleaching Effect as a Result of Immobilisation

The bleaching effect as a result of immobilisation of HRP compared favourably with the studies using free HRP. After 10 min, in excess of 80% of the colour was removed from Chicago Sky Blue with vanillin as accelerator. The PA HRP however, did not prove as effective as either the native or EG HRP. A possible explanation for this is the increased number of attachment points for PA HRP, and this may somewhat restrict access to the active site. These experiments were carried out at room temperature, but, as the immobilised HRP has been shown to be more thermostable than free HRP, it is plausible that they would perform better than the free HRP at elevated temperatures. They would also have the advantage of being reusable so as make them more cost-effective.

9.12.3.5 Effect of Solvents on Bleaching

The bleaching capacity of the native and modified HRP were tested in the presence of two solvents, DMF and dioxane. Vanillin was the accelerator and, as in the 37°C experiments, Chicago Sky Blue was the most easily bleached dye. There was little or no bleaching for Acid Red, Methyl Orange and Direct Blue in the presence of either of the two solvents. The bleaching effect on csb was almost as good as in the absence of solvents. EG HRP was better than the native, removing 87% and 67% of the colour in the presence of DMF and dioxane respectively. This compared with values of 57% and 62% for the native. The PA HRP was as efficient as the native in the presence of the solvents.

9.12.3.6 Effect of Successive additions of Dye on Bleaching with Immobilised HRP (Cycles of Bleaching)

A number of cycles of bleaching were carried out on both free and immobilised HRP. This entailed adding aliquots of dye and hydrogen peroxide to the bleaching system at set intervals. As can be seen in Table 8.6, the immobilised enzymes were much more efficient and continued to remove more colour than the free HRP over the cycles of addition. The free HRP was not able to cope with the extra dye load but it seemed to pose no serious problem for the

was not able to cope with the extra dye load but it seemed to pose no serious problem for the immobilised HRPs. After 5 cycles, the EG HRP had removed more than 60% of the colour while the free EG HRP could only remove 18% of the colour at this stage.

Conclusion

The objective of the project was to produce a chemically stabilised derivative of HRP for use in industrial and environmental situations. This was successfully achieved with some of the chemical reagents used, namely, the phthalic anhydride (PA) and the EG-modified recombinant HRP. A dramatic increase in stability was seen in the case of the PA HRP. The stability properties were explored in full. The unfolding and kinetic properties were also explored in depth. The chemical modification procedures that produced stabilised derivatives affected the lysine residues of the polypeptide only. Immobilisation took place via the carboxylic groups of Asp and Glu residues. The stabilising modifications should be equally applicable to recombinant HRP (if this could be obtained in quantity). Immobilisation could also take place via the carbohydrate of plant HRP. These immobilised derivatives have definite potential in the treatment of wastewaters containing phenolic or coloured compounds. The use of chemically stabilised derivatives of HRP has not been exploited to its full potential in waste treatment applications. It is hoped that the work presented here will aid scientists studying HRP and technologists seeking to cope with pollution problems.

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APPENDIX

List of Publications

O'Brien, A. M. And O'Fagain, C. (1996) Chemical stabilisation of recombinant horseradish peroxidase. Biotechnol. Tech. Dec. 905-910.