The preparation and characterisation of novel microperoxidase biocatalysts

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THE PREPARATION AND CHARACTERISATION OF NOVEL MICROPEROXIDASE BIOCATALYSTS

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ABSTRACT

Microperoxidases are haem oligopeptides, derived from the proteolytic digestion of cytochrome c, possessing peroxidase activity. They are termed "microperoxidases" in contrast to other relatively large peroxidases such as horseradish peroxidase.

This study investigates

i) the preparation of microperoxidases,

ii) some of the properties of microperoxidases including their pH profile and stability, temperature profile, and some of their reactions,

iii) the immobilisation of microperoxidases.

Throughout the study comparisons are drawn between the two different microperoxidases prepared in the study, a microperoxidase purchased from Sigma Chemicals Ltd, horseradish peroxidase, cytochrome c and haemoglobin.

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1.0.0 INTRODUCTION

1.1.0 Peroxidases

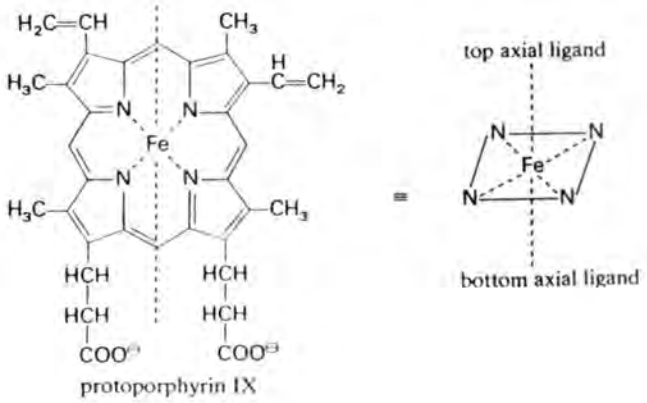
The term peroxidase in the widest sense includes a group of specific enzymes such as NAD peroxidase, fatty acid peroxidases, glutathione peroxidase as well as a group of very non-specific enzymes from different sources simply known as peroxidases (donor: H_2O_2 - oxidoreductase E.C. 1.11.1.7) (Putter J. 1974, Putter J. and Becker R. 1983).

With very few exceptions the typical peroxidases belonging to group E.C.1.11.1.7 are haemoproteins (Putter J. 1974, Putter J.and Becker R. 1983). The first link between peroxidase activity and a chemical structure appeared in 1931 when Kuhn et al found proportionality between activity and light absorption in the Soret band (Paul K.G. 1963).

Most, if not all, haem proteins may be converted to forms which show some peroxidase activity. For example metmyoglobin and methaemoglobin and fragments of non-peroxidase haem proteins such as microperoxidase from cytochrome c are catalytically effective. Even protein free haem complexes show significant peroxidase and catalase-like activity (Frew J.E. and Jones P. 1984, Takashi K. et al 1986).

Haemoproteins contain protohaem or a very closely related haem as a prosthetic group, which forms the active centre of the molecule as far as its biological activity is concerned (Maehly A.C. 1952, Kotani M. 1964).

Fig.1 The Iron Porphyrin Prosthetic Group of Haem Proteins



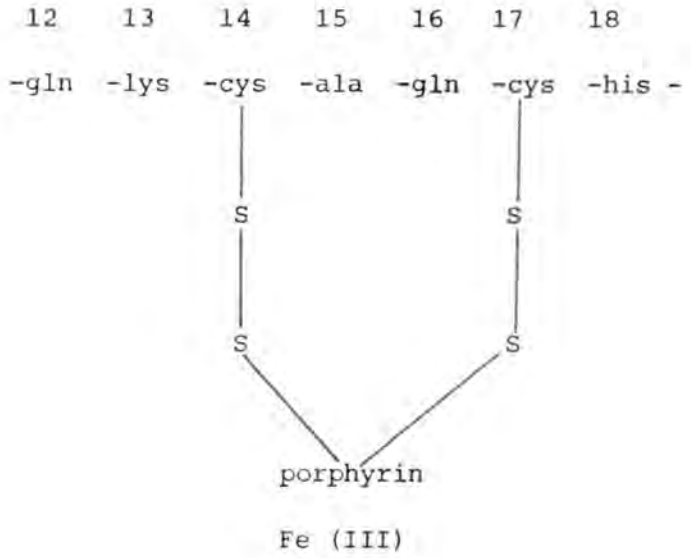
(Walsh C. 1979)

The iron atom of the prosthetic haem group is coordinated to a macrocyclic tetrapyrrole ring (protoporphyrin IX) (Fig. 1). The porphyrin ring is considered to be essentially planar. The fifth and the sixth co-ordinations of the iron atom, which are directly perpendicular to the porphyrin plane, are coupled by nitrogens or other atoms or molecules. Generally at least the fifth (bottom) axial position is filled by a ligand from the apoprotein, the top axial position may be filled by a ligand from the protein, water, oxygen, carbon monoxide, halide ion or some other molecule. In the case of cytochromes both axial positions are occupied by the nitrogen atom in the imidazole ring of histidine contained in the polypeptide chain of the protein. In myoglobin and haemoglobin the fifth position only is occupied by an imidazole ring, the sixth position being occupied by a water

molecule, halogen atom or other molecule or radicle. There is no firm evidence of the identify of the sixth ligand in native horseradish peroxidase. It may be a very tightly bound water molecule or a weak-field protein side chain e.g. carboxylate. The sixth ligand of horsradish peroxidase appears to constrain the porphyrin ring to a more planar conformation, facilitating peroxidase activity (Paul K.G. 1963, Kotani M. 1964, Rakshit G. and Spiro T.G. 1974, Walsh C. 1979, Poulos T.L. and Kraut J. 1980 a).

In horseradish peroxidase and the oxygen-carrying haem proteins (haemoglobin and myoglobin), the iron-porphyrin is a non-covalently bound prosthetic group. However, the single haem prosthetic group of cytochrome c is covalently attached by thioether bonds, formed by the addition of the sulphydril groups of two cysteinyl residues in positions 14 and 17, across the vinyl side chain of the porphyrin ring (Fig. 2) (Paul K.G. 1955, Margoliash E. and Walasek O.F. 1962, Bendall D.S. et al 1971, Rakshit G. and Spiro T.G. 1974, Wilson M.T. et al 1977).

Fig. 2 The Covalently Attached Porphyrin of Cytrochrome c.



1.2.0 The Peroxidase Reaction Mechanism

Peroxidases are widely distributed throughout the biological world. They are found in many plants, in some animal tissues and in many microorganisms. They carry out a wide variety of biosynthetic and degradative functions, calling for the use of peroxides, especially hydrogen peroxide, as an oxidant.

The overall net reaction catalysed by many peroxidases may be simply written as (eq.1) $ROOH + AH_2 \rightarrow ROH + H_2O + A$

eq.1

Where ROOH = hydrogen peroxide or organic hydroperoxide, AH_2 = donor molecule. (Theorell H. 1950, Walsh C. 1979, Poulos T.L. and Kraut J. 1980 a).

In the classical peroxidase reaction the specificity for the peroxide substrate is very high. Only hydrogen peroxide and compounds having the group -O-OH eg acetyl-, methyl-, cumene hydroperoxide can act as substrates. The highest activity is shown towards hydrogen peroxide with a graded activity towards alkyl hydrogen peroxides. The specificity for the donor however is very low and a large number of phenols, aminophenols, diamines, indophenols, leucodyes, ascorbate and several amino acids may be used. (Table 1). Different peroxidases react preferably with some hydrogen donors and not with others, and there may be different pH optima with different donors for the same peroxidase (Chance B. 1950, Theorell H. 1950, Maehly A.C. 1955, Putter J. 1974, Bergmeyer H.U. et al 1983, Putter J. and Becker R. 1983).

There are many common features in the reaction mechanism of peroxidases. The peroxidase reaction consists of two successive steps each involving one hydrogen or electron, but the reaction normally occurs in three distinct stages (eq. 2 to 4).

$$P + ROOH \longrightarrow \text{compounds I} + ROH \qquad \text{eq.2}$$

$$\text{compound I} + AH_2 \longrightarrow \text{compound II} + A \qquad \text{eq.3}$$

$$\text{compound II} + AH_2 \longrightarrow P + A \qquad \text{eq.4}$$

TABLE 1 HYDROGEN DONORS FOR PEROXIDASES

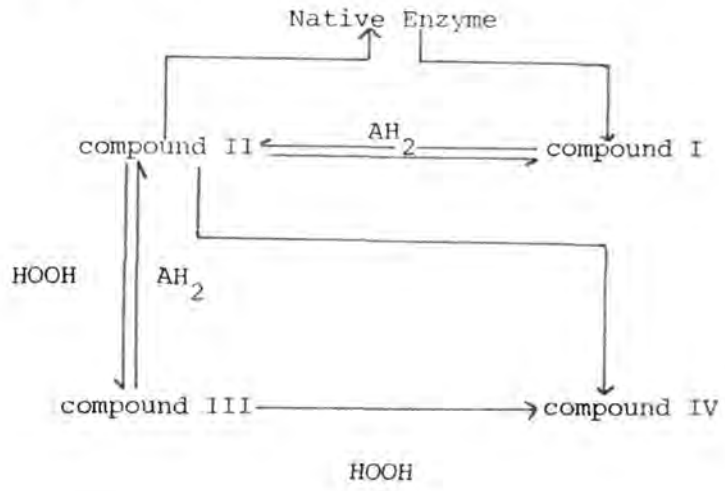
CLASS	COMPOUND
Phenols	p-hydroxyphenol
	hydroquinone
	hydroquinonemethylether
	catechol
	catecholmethylether
	resorcinol
	pyragallol
	guaiacol
	2-cresol
Amines	o-phenylenediamine
	m-phenylenediamine
	aniline
	p-aminobenzoic acid
	o-toluidine
	benzidine
	o-dianisidine
Enediols	reductone
	dihydroxymaleic acid
	ascorbic acid
Miscellaneous	uric acid
	leucomalachite green
	nitrite
	DPNH
	NADH
	azodyes

Where P = native peroxidase, ROOH = hydrogen peroxide or organic hydroperoxide, compound I = intermediate two oxidising equivalents above the native peroxidase, compound II = intermediate one oxidising equivalent above resting state.

Poulos and Kraut (1980) propose that all peroxidases utilise the same mechanism in the first step. The acid-base catalysis of the distal histidine and charge stabilisation promote the heterolysis of the RO-OH bond to produce an oxidised enzyme intermediate two oxidising equivalents above normal (Maehly A.C. 1955, Putter J. 1974, Walsh C. 1979, Poulos T.L. and Kraut J. 1980 a, Poulos T.L. and Kraut J. 1980 b, Putter J. and Becker R. 1983, Frew J.E. and Jones P. 1984).

Where peroxidases differ most notably is in the type of substrate employed as the donors in steps 2 and 3 of the reaction cycle (eq 3 and 4). Thus a general equation cannot be formulated for peroxidase catalysed reactions because the course of the reaction depends on the type of substrate. In the simplest case the same molecule acts as the hydrogen donor for both steps (Poulos T.L. and Kraut J. 1980 a, Putter J. and Becker R. 1983).

Fig. 3 Scheme for Peroxidase Mechanism



(Paul K.G. 1963)

It is debatable whether the mechanistic scheme for horseradish peroxidase can be extended to other peroxidases (Morrison M. and Schonbaum G.R. 1976).

1.2.1 Compound I of the Peroxidase Mechanism

The central feature of the catalytic mechanism is the formation of a spectroscopically distinct intermediate by the action of the oxidising substrate on the native enzyme. This intermediate, known as compound I, was first detected by Theorell and it was initially assumed to be an enzyme-substrate complex (eq. 5).

$$E + H_2O_2 \longrightarrow E(H_2O_2) \qquad \text{eq.5}$$

Evidence however has shown that compound I is a derivative of the enzyme retaining both oxidising equivalents of the peroxide

molecule (eq.6).

$$Fe(III) + ROOH \longrightarrow Fe(V) + ROH \qquad \text{eq.6}$$

The formal oxidation state of compound I is Fe(V) if all the electron deficiency is localised at the iron, but the evidence is overwhelmingly in favour of an oxidation state of Fe(IV) and a cation radicle. The unpaired electron in the tetrapyrrole macrocycle is highly delocalised and contributes to the overall stability of compound I. Compound I of horseradish peroxidase is bright green (Maehly A.C. 1955, Schonbaum G.R. and Lo S. 1972, Cotton M.L. and Dunford H.B. 1973, Rakshit G. and Spiro T.G. 1974, Davies D.M. et al 1976, Morrison M. and Schonbaum G.R. 1975, Jones P. et al 1977, Walsh C. 1979, Poulos T.L. and Kraut J. 1980 a and 1980 b, Roberts J.E. et al 1981, Rutter R. and Hager L.P. 1982, Adediran S.A. and Dunford H.B. 1983, Kedderis G.L. and Hollenberg P. F. 1983, Frew J.E. and Jones P. 1984, Hashimoto S. et al 1986, Nakatani H. and Takahashi K. 1986, Edwards S.L. et al 1987).

1.2.2 Compound II

Compound II is a second enzyme intermediate in the peroxidase mechanism. It is produced when compound I is reduced by one electron. The formal oxidation state of compound II, which is one oxidising equivalent above the native enzyme is Fe(IV). Mossbauer, ENDOR and resonance Raman spectra studies agree that compound II contains the ferryl haem with an oxene ligand Fe(IV) = O. Compound II of horseradish peroxidase is red (Walsh C. 1979, Frew J.E. and Jones P. 1984, Hashimoto S. et al 1986).

1.2.3 Compound III

Compound III is an oxygen adduct formed in the presence of excess hydrogen peroxide. Compound III (oxyperoxidase) may be obtained either when oxygen reacts with ferrous (FeII) peroxidase or when the superoxide radical $O_2^{-\cdot}$ is added to ferric (Fe III) peroxidase.

Compound III decays to the ferric peroxidase through a mechanism depending on the presence or absence of free ferrous peroxidase. The decay is slow but accelerated in the presence of ferrous peroxidase, indicating the co-oxidation of ferrous iron with the decay of compound III. The superoxide radicle is formed during the decay of compound III. Horseradish peroxidase is slowly destroyed under conditions where compound III prevails (Chance B. 1950, Paul K.G. 1963, Rotilio G. et al 1974, Frew J.E. and Jones P. 1984).

Compound III is involved in the hydroxylation of aromatic compounds by peroxidases (section 4.14.0) and in the oxidation of horseradish peroxidase with dihydroxymaleic acid under aerobic conditions (Chance B. 1950, Theorell H. 1950, Halliwell B. and Ahluwalia S. 1976, Halliwell B. 1977, Dordick J.S. et al 1986).

1.2.4 Compound IV

The formation of compound I to compound III is reversible. Compound IV appears after prolonged exposure to peroxide and represents an irreversible change, probably an attack of the peroxide on the porphyrin ring. It has long been known that hydrogen peroxide damages haem groups causing a decrease in the

Soret absorbance (Paul K.G. 1953, Bodaness R.S. 1983, Rice H. R. et al 1983).

1.3.0 The Uses of Peroxidases

Peroxidases catalyse the oxidation of a great number of organic compounds at the expense of peroxide. Although the normal peroxidase reaction consists of the transfer of hydrogen or electrons from a donor to hydrogen peroxide there are examples of peroxidases acting like oxidases (eq. 7) and mono-oxygenases (eq. 8) but with a different mechanism.

$$SH_2 + O_2 \rightarrow S + H_2O_2 \qquad \text{eq.7}$$

$$S\text{-}H + O_2 + NADPH \longrightarrow S\text{-}OH + NADPH^+ + OH^- \qquad \text{eq.8}$$

The peroxidase dependent oxygen transfer from hydrogen peroxide to thioanisoles has been demonstrated. Peroxidases are known to Catalyse N- and O- dealkylations (section 4.13.0) hydroxylations (section 4.14.0) and polymerisation reactions. (Putter J. 1974, Halliwell B. and Ahluwalia S. 1976, Halliwell B. 1977, Galliani G. et al 1978, Griffin B.W. and Ting T.L. 1978, Hewson W.D. and Lager L.P. 1979, Kedderis G.L. et al 1980, Kedderis G.L. and Hollenberg P.F. 1983, Putter J.and Becker R. 1983, Meunier G. and Meunier B. 1985, Neidleman S.L. and Geigert J. 1985, Dordick J.S. et al 1986, Kobayashi S. et al 1986).

1.3.1 Peroxidases in Waste Removal

Horseradish peroxidase catalyses the oxidative coupling of dissolved phenols and aromatic amines in waste streams. In the course of the oxidation, the corresponding phenolic and aromatic

amine radicals are generated. These radicals diffuse from the active centre of the enzyme into the solution where they polymerise to non-toxic polyaromatic products. The resulting high molecular weight compounds are less water soluble than the initial phenols and aromatic amines and can be removed by sedimentation or filtration.

Certain phenols and aromatic amines cannot be enzymatically precipitated by peroxidases however the easily removed compounds aid the precipitation of less readily removed pollutants by forming high molecular weight mixed polymers. There is a relationship between the structure of pollutant and the efficiency of enzymatic precipitation. Electron donating substituents (eg. methyl-or methoxy- groups) at the m-position favour removal to a greater extent than those in the o- or p-positions. The exact converse is true for electron pulling groups (e.g. chloride). An increase in hydrophobicity markedly improves the removal efficiency (Galliani G. et al 1978, Klibanov A.M. et al 1980, Alberti B.N. and Klibanov A.M. 1981, Klibanov A.M. and Morris E.D. 1981, Sawahata T. and Neal R.A. 1982, Hopkins T.R. 1984, Maloney S.W. et al 1984, Chapsal J.M. et al 1986).

Over forty different phenols and aromatic amines including human carcinogens and mutagens have been removed from water using peroxidases, the efficiency of removal in many cases exceeding 99%. However, although the peroxidase/hydrogen peroxide system is effective in eliminating chlorinated phenols at very low levels from drinking waters, the products are not removed from the water (Alberti B.N. and Klibanov A.M. 1981, Maloney S.W. et al 1983).

Hydrogen peroxide is expensive and short lived in a real waste stream situation. Hopkins (1984) found that water soluble aromatic amines may be efficiently and economically removed from water using a peroxidase, alcoholic oxidase and a straight chain C_1-C_4 alcohol or glucose oxidase and glucose. In these systems hydrogen peroxide is produced continuously in situ avoiding hydrogen peroxide storage instability problems. The process can be immobilised and loaded in a flow reactor through which the fluid to be treated is then passed, allowing the benefit of continuous treatment and the reuse of the enzyme (Hopkins T.R. 1984).

Horseradish peroxidase is one of the most active enzymes known, therefore only very small quantities, which need not be purified, are required for the treatment of waste waters, making it a potentially cheap method of removing toxic and carcinogenic aromatic compounds from water. Haemoglobin, the major constituent of blood is a cheap and abundant protein possessing peroxidase activity, which seems to act on the same range of substrates as horseradish peroxidase. It has been reported that immobilised blood haemolysate and hydrogen peroxide resulted in essentially complete removal of benzidine and o-dianisidine (carcinogenic aromatic amines) in a shorter time than horseradish peroxidase (Chapsal J.M. et al 1986).

The peroxidase treatment of waste streams is much less sensitive than bacterial degradation to variations in pH, other toxic constituents, concentration of phenol or aromatic amine and temperature (Klibanov A.M. et al 1983).

The peroxidase/hydrogen peroxide systems can also remove

the colour (due to the products of lignin chlorination and oxidation) in the effluent from bleach plants. There is no precipitation in the decolorisation reaction (Paice M.G. and Jurasek L. 1984).

Peroxidases/hydrogen peroxide also oxidise halide salts or phenolic compounds in industrial process streams producing oxidation products toxic to micro-organisms. Quinones or hydroquinones toxic to micro-organisms, are produced by the oxidation of phenolic compounds by peroxidases. The antimicrobial activity is a function of the type of quinone produced and the degree of phenolic polymerisation. Pulp and papermill process streams include phenolic derivatives derived from lignin, catechol, guaiacol, acid lignin, black liquor, resorcinol are good substrates for the system (Orndoff S. 1984).

1.3.2 Peroxidases in Clinical Assays

Due to the high turnover rate, good stability, ease of assay and relatively low molecular weight, peroxidases have become important enzymes in the colorimetric determination of biological materials and are widely used as the marker enzymes in immunoassays (Ngo T.T. and Lenhoff H.M. 1980, Shinmen Y. et al 1986).

The peroxidase activity is generally monitored by the formation of a coloured compound from a colourless oxygen acceptor (eq. 9).

H_2O_2 + Colourless acceptor $\rightarrow$ $2H_2O$ + coloured reaction product

eq.9

Glucose, cholesterol, uric acid and galactose can be measured by

peroxidase coupled reactions (eg. eq.10 to eq.11).

Uric acid $\xrightarrow{\text{uricase}}$ allantoin + CO_2 + H_2O_2 eq.10

H_2O_2 + 3, methyl-2-2-benzothiazidine + N,N-dimethyl-aniline $\xrightarrow{\text{peroxidase}}$ indamine pigment +H_2O eq.11

(Ngo T.T. and Lenhoff H.M. 1980, Yamada Y. et al 1987).

The enzyme catalysed processes which lead to the formation of hydrogen peroxide can be coupled to chemiluminescence detection. Light emission by chemiluminescent systems is an alternative to radiolabelling. The conditions of the enzymic process are controlled so that the amount of hydrogen peroxide generated is proportional to the concentration or activity to be measured. The hydrogen peroxide is then allowed to react with excess chemiluminescent generating reagents so that the resulting chemiluminescent intensity is proportional to the concentration of peroxide (eq. 12 to 13).

β-D-glucose + O_2 $\xrightarrow[\text{oxidase}]{\text{glucose}}$ D-gluconolactone + H_2O_2 eq.12

H_2O_2 + luminol $\xrightarrow{\text{peroxidase}}$ chemiluminescence eq.13

Luminol and hydrogen peroxide will not react to any significant extent unless a peroxidase is present. Among the oxidation systems for chemiluminescent compounds, hydrogen peroxide-microperoxidase and hydrogen peroxidase - haematin systems provide the most sensitive assays. The latter system is more difficult to use because the activity of haematin solutions vary with age, it is more expensive and less soluble. Luminol is most effective over the pH range 10-11, thus microperoxidase which has an alkaline pH otpimum (section 3.6.0) is a suitable peroxidase

(Seitz W.R. 1978, Schroeder H.R. et al 1978).

1.3.3 Peroxidases as Electron Microscope Tracers

Molecules possessing peroxidase activity have found widespread usefulness as tracers in electron microscopy studies by the simplicity of performing the histochemical reactions for their detection.

Horseradish peroxidase, myoglobin and cytochrome c have commonly been used, however they have a Stokes radius of 15A° and their ability to probe permeability barriers whose pore size is below this range is limited. Their relatively large size may also alter the biological activity of smaller ligands to which they may be coupled.

The haem oligopeptides obtained by proteolytic digestion of cytochrome c are attractive as electron microscopy tracers. They retain their peroxidase activity enabling them to be easily located, they have a Stokes radius of less than 15A°, extending the range of pore detection in permeability studies and have less effect on the biological activity of attached ligands. The haem oligopeptides are simple and inexpensive to prepare, and they may be used in in-vivo studies with no detrimental effects (Kraehenbuhl J.P. et al 1974, Plattner H. et al 1977).

1.3.4 Protein Cross-Linking by Peroxidases

Enzyme catalysed reactions which cross-link proteins, altering their solubility and other functional properties are potentially useful in modifying the functional properties of food

proteins. Enzyme catalysed reactions known to cross-link proteins include peroxidases. The treatment of proteins with hydrogen peroxide and peroxidase causes oxidation of tyrosine to di- and tetratyrosine in proteins. It has been reported that the addition of peroxidase and hydrogen peroxide improves the dough forming properties and baking performance of wheat flours. (Matheis G. and Whitaker J.R. 1984).

Addition of low molecular weight hydrogen donors (phenolic or diamine compounds) to the protein, hydrogen peroxide and peroxidase system causes cross-linking of proteins via an oxidative deamination. The cross-linking and labelling of proteins on peroxide treatment does not occur in the absence of haem proteins (Rice H.R. et al 1983).

The microperoxidase haem undecapeptide, derived from cytochrome c by proteolytic digestion, has been used to study the alterations in lens crystallin polypeptides similar to those changes found in cataracts (Bodaness R.S. et al 1984).

1.3.5 The Uses of Microperoxidases

The haem oligopeptides derived from the proteolytic digestions of cytochrome c offer the possibility of studying the reactions of the haem group relatively unshielded by protein yet in a form soluble over a wide range of pH. The haem oligopeptides or microperoxidases have been used as models to establish the role of protein in peroxidase activity (Blumenthal D.C. and Kassner R.J. 1980, Baldwin D.A. et al 1985), as models to study ligand binding (Wilson M.T. et al 1977, Blumenthal D.C. and Kassner R.J. 1979,

Blumenthal D.C. and Kassner R.J. 1980, Peterson J. and Wilson M.T. 1980, Clore G.M. et al 1981, Peterson J. et al 1983), as well as electron microscope tracers (Kraehenbuhl J.P, et al 1974, Plattner H. et al 1977) and to study protein cross-linking (Bodaness R.S. et al 1984).

There are very few references to the properties of micoperoxidases. Most of the work so far has been concerned with microperoxidases in academic studies (e.g. as models for kinetic studies) and as tracers for analytical work. There is very little mention of microperoxidases in the biotechnology field.

This study was undertaken to identify some of the properties of microperoxidases for use as biocatalysts for the production of fine chemicals, and as a very versatile catalyst for different reactions, such as halogenation and demethylation reactions.

2.0.0 MATERIALS AND METHODS

Microperoxidase mp-11, horseradish peroxidase type II (E.C. No. 1.11.1.7), cytochrome c type III (from horse heart), haemoglobin (from bovine blood), guaiacol, monochlorodimedone, N,N - dimethylaniline, 2,4 - pentanedione, tetrazotised o-dianisidine, pepsin (E.C. No. 3.4.23.1), trypsin (E.C. No. 3.4.21.4), protease type XXVII (nagarase) and diphenyl carbarmyl chloride were purchased from Sigma Chemicals Limited. The hydrogen peroxide was obtained from Fisons, the solvents from BDH (Analar), Aldrich and Rathburn Chemicals Limited, polyethylene glycol was from Aldrich. The Sephadex gel filtration supports and PD-10 columns were from Pharmacia. The Macrosorb K (GA) immobilisation support was obtained from Sterling Organics Limited, the UOP support from UOP Biological Products, and the controlled pore glass beads from Sigma Chemicals Limited. All other chemicals were from Sigma Chemicals Limited and BDH.

All spectrophotometric measurements were made using a Kontron Uvikon 860 spectrophotometer, and unless otherwise stated all assays were carried out at room temperature in 3 ml plastic cuvettes with a path length of 1.0 cm.

Throughout the experiments the solutions of microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin and the proteolytic digests of cytochrome c were kept on ice or stored in the cold room (4°C).

Fresh solutions of hydrogen peroxide were prepared daily, guaiacol solutions were kept for a minimum of three days.

2.1.0 The Peroxidase Assay

Throughout the series of experiments the peroxidase activity of microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin and proteolytic digests of cytochrome c were assayed using the guaiacol assay (Chance B. and Maehly A.C. 1955).

The conditions of the assay were varied slightly from experiment to experiment, but the basic assay was

2.90 ml buffer

0.05 ml 20mM guaiacol

10-50 µl haem protein solution of required concentration

10 µl 40mM hydrogen peroxide.

The reagents were mixed in situ in 3 ml cuvettes with a path length of 1.0 cm. The reaction was initiated by the addition of the hydrogen peroxide and carried out at room temperature.

The rate of reaction was determined by measuring the increase in absorbance at 470 nm due to the formation of the coloured reaction product tetraguaiacol, using a molar extinction coefficient of $2.66 \times 10^4 \ M^{-1} \ cm^{-1}$. The blank reference cuvette was identical to the sample cuvette but without the addition of hydrogen peroxide.

The peroxide activities of microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin and two proteolytic digests of cytochrome c (section 2.4.0) were assayed in triplicate using 50 mM dipotassium hydrogen phosphate pH 8.0 as the buffer.

The results were expressed in terms of µmol tetraguaiacol formed/min/mg of haem protein and in terms of mol tetraguaiacol formed/min/mol haem.

2.1.1 The Effect of Substrate Concentration on the Guaiacol Assay

The effect of the concentration of the substrate, hydrogen peroxide, on the assay was determined. The assay was

2.90 ml 20 mM dipotassium hydrogen phosphate buffer pH 7.0

0.05 ml 20 mM guaiacol

10 µl 0.1 mg/ml microperoxidase mp-11

10 µl x mM hydrogen peroxide.

The assays were carried out in triplicate for a range of concentrations (10-100mM) of hydrogen peroxide and a graph of average activity (in µmol tetraguaiacol formed /min/mg microperoxidase mp-11) vs. concentration of hydrogen peroxide (in mM) was plotted.

2.1.2 The Effect of Peroxidase Concentration on the Guaiacol Assay

The effect of the concentration of peroxidase on the assay was investigated by varying the concentration of microperoxidase mp-11 used in the assay. The assay was carried out in triplicate as follows:

2.90 ml 50mM tris - HCl buffer pH 8.0

0.05 ml 20 mM guaiacol

10 µl ymg/ml microperoxidase mp-11

10 µl 40mM hydrogen peroxide to initiate the reaction.

A graph of average activity (in µmol tetraguaiacol formed/min/mg) vs. the concentration of microperoxidase mp-11 (in mg/ml) was plotted.

2.2.0 Proteolytic Digestions of Cytochrome c and Haemoglobin

Cytochrome c type III (from horse heart) and haemoglobin (from bovine blood) were digested using four different proteolytic procedures.

2.2.1 Pepsin Digestion

2.5 mg cytochrome c or haemoglobin were dissolved in 5.0 ml distilled water. The pH was adjusted to 2.6 using very dilute hydrochloric acid. A 0.1 ml aliquot of 10 mg/ml pepsin solution was added.

2.2.2 Protease type XXVII (Nagarase) Digestion

2.5mg cytochrome c or haemoglobin were dissolved in 5.0 ml distilled water and the pH adjusted to 7.5 using very weak hydrochloric acid. 0.1 ml aliquot of 10 mg/ml protease type XXVII was added.

2.2.3 Trypsin Digestion

2.5 mg cytochrome c or haemoglobin were dissolved in 5.0 ml 0.5M sodium bicarbonate solution pH 8.0. A 0.1 ml aliquot of 10 mg/ml trypsin solution was added.

2.2.4 DPCC - Trypsin Digestion

2.5mg cytochrome c or haemoglobin were dissolved in 5.0 ml 0.5M sodium bicarbonate solution pH 8.0. A 10 mg/ml solution of trypsin was treated with a small amount of diphenyl carbarmyl chloride (DPCC) which inhibits any residual chymotrypsin activity. A 0.1 ml aliquot of the resulting solutions was added to the digestion mixture.

All digestions were incubated at 27°C (in a water bath) and at suitable time intervals a 30 µl aliquot was removed and assayed for peroxidase activity. The assay was

2.90 ml 20 mM dipotassium hydrogen phosphate

pH 7.0

0.05 ml 20 mM guaiacol

30 µl 0.5 mg/ml digestion mixture

10 µl 40mM hydrogen peroxide.

The results were expressed as µmol tetraguaiacol formed/min/mg cytochrome c or haemoglobin.

The pepsin digest of haemoglobin was repeated, assaying for peroxidase activity at 5 min intervals for 75 min.

2.3.0 The Gel Filtration of Pepsin Digested Cytochrome c

These experiments were designed to separate digested cytochrome c from undigested cytochrome c in the digestion mixture using gel filtration and to try to correlate the peroxidase activity of the cytochrome c fragments with the size of the fragments.

The pepsin digest was chosen for the investigation because the enzyme pepsin is inactivated at alkaline pH, therefore the digestion is readily terminated by using an alkaline buffer with the gel.

2.3.1 Preparation of Gel Filtration Columns

Sephadex G15, G25 and G50 were washed with 50mm tris-HCl buffer pH 8.0 to remove any fines and swollen overnight at 4°C in fresh buffer. The excess buffer was removed to leave a slurry of approximately 75% solids which was degassed.

The following columns were prepared:

0.5 cm x 10 cm Sephadex G15

0.5 cm x 10 cm Sephadex G25

1.0 cm x 30 cm Sephadex G50

1.5 cm x 75 cm Sephadex G50.

The columns were equilibrated with degassed 50mM tris-HCl buffer pH 8.0. A peristaltic pump with a flow rate of 1.3 ml/min was used.

2.3.2 Preliminary Investigation into the Separation of Digested and Undigested Cytochrome c

Preliminary experiments were carried out to determine the size of the column required to separate digested cytochrome c from undigested cytochrome c in a mixture.

Pepsin digested cytochrome c, which had been previously prepared and lyophilised, was dissolved in distilled water to give a concentration of 5 mg lyophilised powder/ml. A 200 µl sample

containing 100 µl each 5 mg/ml pepsin digested cytochrome c and 5 mg/ml cytochrome c was loaded onto each column and the samples eluted with degassed 50mM tris - HCl buffer pH 8.0 (flow rate 1.3 ml/min). Fractions of approximately 3ml were collected by hand and the elution of the haem proteins followed by measuring the spectra of the fractions (in quartz glass cuvettes). The absorbance in the Soret region (haems show characteristic absorbance peaks in the Soret region) was measured using the peak detection program of the Kontron Uvikon 860 and graphs of absorbance vs. time of elution (in minutes) plotted where applicable.

The experiment was repeated to separate a mixture of 100 µl each 4.5 mg/ml pepsin digested cytochrome c and 4.5 mg/ml cytochrome c on Sephadex G50 (1.5 x 75 cm) using a dissociating buffer of 50 mM tris-HCl and 3M urea pH 8.0.

The fractions were collected and scanned as before. Fractions with a positive peak in the Soret region were assayed for peroxidase activity as follows:

2.90 ml 50mM tris-Hcl pH 8.0

0.05 ml 20mM guaiacol

30 µl eluted fraction

10 µl 40mM hydrogen peroxide.

The activity was expressed as mol tetraguaiacol formed/min/mol haem. Graphs of absorbance vs. time of elution (min) and activity of fraction (in mol/min/mol haem) vs. time of elution (min) were plotted.

2.3.3 The Gel Filtration of Pepsin Digested Cytochrome c After Different Times of Digestion

A pepsin digestion of cytochrome c was set up. 50 mg cytochrome c were dissolved in 9.0 ml distilled water and the pH adjusted to 2.6 using very dilute hydrochloric acid. 1.0 ml 10 mg/ml pepsin solution was added and the mixture incubated in a waterbath at 27°C.

At 1 hr intervals 0.5 ml aliquots were removed and frozen in Eppendorf tubes at -20°C to stop the digestion. At suitable time intervals the frozen samples were thawed and 200 µl loaded on to a Sephadex G50 (1.5 cm x 7.5 cm) column and eluted with degassed 50 mM tris-HCl/3M urea buffer pH 8.0 (flow rate 1.3 ml/min).

The eluted fractions were collected at 2 min intervals using an automatic fraction collector and scanned in quartz glass cuvettes. Fractions with a positive Soret band were assayed for peroxidase activity as before (section 2.3.2).

The experiment was repeated taking 0.5 ml samples from the digestion mixture at 5 min intervals for 50 min. The fractions were collected and scanned as before and the absorbance in the Soret region and at 280 nm were measure using the peak detection program of the Kontron Uvikon 860. As tris-HCl buffer interferes with the absorbance at 280 nm, 50 mM dipotassium hydrogen phosphate/3M urea buffer pH 8.0 was used to elute the fraction.

The peroxidase activity of fractions with a positive soret band were assayed as in section 2.3.2. Activities were expressed in mol tetraguaiacol formed/min/mol haem.

Graphs of absorbance of fraction vs. time of elution (min)

and activity of fraction vs. time of elution were plotted for each sample.

2.3.4 The Gel Filtration of Pepsin Digested Cytochrome c using PD-10 Columns

PD-10 columns are small (bed volume 9.1 ml) prepacked columns of Sephadex G25 generally used for desalting proteins. The columns are quick and easy to run.

A PD-10 column was equilibrated with 25 ml 50 mM dipotassium hydrogen phosphate buffer pH 8.0. 200 µl pepsin digested cytochrome c and 2.3 ml 50 mM dipotassium hydrogen phosphate pH 8.0 were loaded on to the column and allowed to soak in. The column was washed with the same buffer until the whole sample had been eluted from the column.

Fractions (2.5 ml) were collected by hand and scanned in quartz glass cuvettes. The peak height of the Soret band was measured using the peak detection program of the Kontron Uvikon 860. The peroxidase activity of the fractions was measured (as in section 2.3.2). The specific activity (in mol/min/mol haem) and the total activity (in mol/min) were calculated for each fraction and a graph of activity vs. fraction number was plotted.

The experiment was repeated using a second PD-10 column equilibrated with 50 mM dipotassium hydrogen phosphate/3M urea buffer pH 8.0 and using the dissociating buffer to elute the fractions.

2.3.5 Investigation into the Shift of the Soret Band of the Fractions Eluted from the Column: The Effect of Hydrogen Peroxide and Sodium Dithionite on the Absorption Spectra

A shift in the position of the Soret Band was noticed as the fractions were eluted from the column. The cause of this was investigated.

A 0.1 mg/ml solution of pepsin digested cytochrome c dissolved in phosphate buffer was scanned in a quartz glass cuvette against a blank of phosphate buffer. A 10 µl aliquot of 40 mM hydrogen peroxide was added to the pepsin digested cytochrome c and mixed. The mixture was immediately scanned and scanned repeatedly at 5 min intervals.

The experiment was repeated with the addition of a small amount of sodium dithionite in lieu of hydrogen peroxide.

For each scan the absorbance of the Soret peak was determined using the peak detection program of the Kontron Uvikon 860 spectrophotometer.

2.4.0 The Preparation of Microperoxidases for Further Experiments

Pepsin digested cytochrome c and trypsin digested cytochrome c were prepared for further experiments.

2.4.1 Pepsin Digested Cytochrome c

100 mg cytochrome c were dissolved in 19.0 ml distilled water and the pH adjusted to 2.6 using very weak hydrochloric acid. 1.0 ml 10 mg/ml pepsin was added.

2.4.2 Trypsin Digested Cytochrome c

100mg cytochrome c were dissolved in 19.0 ml 0.5M sodium bicarbonate solution pH 8.0 and 1.0 ml 10 mg/ml trypsin added.

The digestions were carried out at 27°C for approximately 30 hr. The digestion process was terminated by boiling the digestion mixtures for 5 min to destroy the proteolytic enzymes. The digests were frozen rapidly using dry ice and a solvent and lyophilised.

Further digestions were later carried out as above.

2.5.0 An Estimation of the Number of Haems

The object of this experiment was to estimate the number of haems per mg of microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome c, enabling their peroxidase activity to be expressed in terms of mol tetraguaiacol formed/min/mol haem.

Solutions of the haem proteins dissolved in phosphate buffer were scanned in quartz glass cuvettes against a blank of phosphate buffer. The height of the Soret peak was measured using the peak detection program of the Kontron Uvikon 860.

The number of haems/mg haem protein was estimated.

2.6.0 The pH Profiles of Microperoxidase mp-11, Horseradish Peroxidase, Pepsin Digested Cytochrome c and Trypsin Digested Cytochrome c

The peroxidase activity of microperoxidase mp-11 and horseradish peroxidase were assayed in triplicate in buffers of

different pH ranging from 2.6 - 11.8.

The buffers were selected from "Data for Biochemical Research" 3rd Edition 1986 and prepared accordingly. The buffers were:

i) citric acid-disodium phosphate (McIlvaine) buffer pH 2.6 - 7.4.

ii) tris (hydroxymethylaminomethane) buffer pH 7.4 - 8.6

iii) glycine-sodium hydroxide buffer pH 8.6 - 10.4

iv) phosphate buffer pH 11.0 - 11.8.

The buffers were chosen so that the pH of one buffer overlapped with the pH of another buffer, enabling the effect of a particular buffer on the peroxidase activity of microperoxidase mp-11 and horseradish peroxidase to be observed.

It was necessary to vary the volume and the concentration of the microperoxidase mp-11 used in the assay according to the buffer. The results were expressed in terms of μmol/tetraguaiacol formed/min/mg and mol tetraguaiacol formed/min/mol haem. Graphs of average activity vs. pH were plotted.

2.6.2 pH Profile of Microperoxidase mp-11 using Zwitterionic Biological Buffers

A second pH profile was prepared for microperoxidase mp-11 using Zwitterionic biological buffers. The buffers were:

i) 50mM bis tris buffer pH 5.8 - 7.0

ii) 50mM bis tris propane buffer pH 6.5 - 9.5

iii) 50mM tricine buffer pH 7.6 - 8.5.

The pH of the buffers was adjusted with hydrochloric acid

or sodium hydroxide solution accordingly.

The peroxidase activity of microperoxidase mp-11 was assayed in triplicate for each pH and the activity expressed as before (section 2.6.1). A graph of average activity (mol/min/mol haem) vs. pH was plotted for the results from the bis-tris propane buffer.

2.6.3 pH Profiles of Pepsin Digested and Trypsin Digested Cytochrome c

The peroxidase activity of the two proteolytic digests of cytochrome c (prepared in section 2.4.0) were assayed in triplicate in buffers of different pH. The buffers were:

i) 50mM acetate buffer pH 3.0 - 5.0 (pH adjusted using hydrochloric acid).

ii) 50mM dipotassium hydrogen phosphate buffer pH 5.5 - 11.5 (pH adjusted using phosphoric acid or sodium hydroxide solution as required).

The results were expressed as μmol tetraguaiacol formed/min/mg lyophilised digest and as mol tetraguaiacol formed/min/mol haem. Graphs of average activity (in mol/min/mol haem) vs. pH were plotted.

2.7.0 The pH Stability of Microperoxidase mp-11 and Horseradish Peroxidase

The stability of microperoxidase mp-11 and horseradish peroxidase in buffers of different pH were determined.

A pH range from 3.0 - 11.0 was prepared using the same

buffers as in section 2.6.1. 1.0 mg microperoxidase mp-11 was dissolved in 1.0 ml of each buffer. A 2.0 mg/ml solution of horseradish peroxidase was prepared and 0.1 ml of this was added to 0.9 ml of each buffer giving a final concentration of 0.2 mg horseradish peroxidase/ml buffer.

The peroxidase activities of microperoxidase mp-11 and horseradish peroxidase were assayed in duplicate at time intervals from time 0 to 1 month. The results were expressed as µmol tetraguaiacol formed/min/ mg.

2.8.0 The Temperature Profiles of Microperoxidase mp-11, Horseradish Peroxidase, Pepsin Digested and Trypsin Digested Cytochrome c

The temperature profiles of microperoxidase mp-11, horseradish peroxidase, pepsin digested cytochrome c and trypsin digested cytochrome c were prepared by measuring their peroxidase activities at different temperatures.

The Kontron Uvikon 860 spectrophotometer was connected to a waterbath so that the temperature of the cell holder could be varied during the experiment. The assay reagents were cooled or warmed to the temperature of the assay by keeping them in the water bath. The haem protein solutions however were kept on ice during the experiment.

The guaiacol assay was carried out in duplicate or triplicate where possible for each temperature (there was some difficulty in maintaining the temperature of the cell holder, particularly at the lower temperatures).

The assays were carried out in buffers previously found to give optimum peroxidase activity (see section 2.6). The buffers used were 50mM bis tris propane pH 8.5, 50mM bis tris propane pH 8.0 and 50mM dipotassium hydrogen phosphate pH 9.0 for microperoxidase mp-11, horseradish peroxidase and the two proteolytic digests of cytochrome c respectively.

The activities were expressed in terms of μmol tetraguaiacol formed/min/mg and mol tetraguaiacol formed/min/mol haem. Graphs of activity (mol/min/mol haem) vs. temperature (°C) were plotted.

2.9.0 Peroxidase Activity in Different Media

The peroxidase activity of microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome c (prepared in section 2.4.0) were assayed in distilled water and a number of water-miscible organic solvents.

The peroxidase activity was assayed using the guaiacol assay, replacing the buffer with an equal volume of distilled water or solvent and the production of tetraguaiacol at 470nm was measured as before (section 2.1.0). Quartz glass curvettes were used to assay the peroxidase activity of the haem proteins in solvents as some of the solvents have a detrimental effect on plastic.

As high concentrations of haem protein solutions (10 mg/ml) were required for the assays in solvents, each assay was only attempted once.

The results were expressed as μmoles tetraguaiacol formed/min/mg and mol tetraguaiacol formed/min/mol haem.

2.10.0 The Catalase Activity of Microperoxidase mp-11, Horseradish Peroxidase, Cytochrome c, Haemoglobin, Pepsin Digested and Trypsin Digested Cytochrome c.

2.10.1 The Spectrophotometric Catalase Assay

Microperoxidase mp-11 and horseradish peroxidase were assayed for catalase activity using the spectrophotometric assay (Chance B. and Maehly A.C. 1955, Aebi H. 1974, Aebi H. 1983). The assay contained:

1.0 ml 50mM dipotassium hydrogen phosphate buffer pH 8.0

2.0 ml 1.0 mg/ml microperoxidase mp-11 or horseradish peroxidase

1.0 ml 30 mM hydrogen peroxide.

The reaction was started by the addition of hydrogen peroxide. The assays were carried out in quartz glass cuvettes, path length 1.0 cm and the decrease in absorbance at 240 nm due to the disappearance of hydrogen peroxide was monitored.

2.10.2 Detection of Catalase Activity by the Evolution of Oxygen

Microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome c were tested for catalase activity by the addition of hydrogen peroxide to a solution of the haem protein and observing for the evolution of oxygen and decolorisation of the solution.

2.11.0 Determination of the Hydroperoxidase Activity of the Haem Proteins

The guaiacol assay (Chance B. and Maehly A.C. 1955) was modified to enable the hydroperoxidase activity of the haem proteins to be measured. The hydrogen peroxide in the assay was replaced by an equimolar amount of cumene hydroperoxide. The assay contained:

2.90 ml 50 mM dipotassium hydrogen phosphate buffer pH 8.0 - 9.0

0.05 ml 20 mM guaiacol

30 µl haem protein solution of required concentration

10 µl 40 mM hydrogen peroxide to initiate the reaction.

The hydroperoxidase activities of microperoxidase mp-11, horseradish peroxidase, cytochrome c and haemoglobin were determined in buffer pH 8.0. The hydroperoxidase activities of the two proteolytic digests were assayed in buffer pH 9.0.

The assays were carried out in triplicate and the rate of reaction was determined (as before) by measuring the increase in absorbance at 470 nm due to the production of tetraguaiacol. The results were expressed in terms of µmol tetraguaiacol formed/min/mg and mol tetraguaiacol formed/min/mol haem.

2.12.0 Halogenation Reactions by Peroxidases

The aim of these experiments was to determine the ability of microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome c (section 2.4.0) to oxidise halide ions to the

molecular species and to halogenate the chloroperoxidase substrate monochlorodimedone.

2.12.1 The Oxidation of Iodide to Iodine

The guaiacol assay for peroxidase activity (Chance B. and Maehly A.C. 1955) was modified by substituting the 20 mM guaiacol with 0.1M potassium iodide.

The assay was

2.90 ml 50 mM dipotasium hydrogen phosphate buffer pH 8.0 or 9.0.

0.05 ml 0.1 M potassium iodide

30 µl haem protein solution of required concentration.

10 µl 40mM hydrogen peroxide to initiate the reaction.

The assays were carried out in triplicate in quartz glass cuvettes. The iodine produced is in equilibrium with the triiodide ion as described by equation 14.

$$I_2 + I^- \longrightarrow I_3^- \qquad \text{eq. 14}$$

The triiodide ion has a molar extinction coefficient of 2.62×10^4 M^{-1} cm^{-1} at 353 nm (Roman R. and Dunford H.B. 1972). The reaction was therefore monitored by measuring the increase in absorbance at 353 nm due to the production of iodine.

The results were expressed in terms of µmol iodine produced/min/mg and mol iodine formed/min/mol haem.

2.12.2 The Oxidation of Bromide to Bromine

The reaction mixture as identical to that for the

oxidation of iodide to iodine (section 2.12.1) using 0.1M sodium bromide in lieu of 0.1M potassium iodide.

The assays were carried out in triplicate in quartz glass curvettes. The rate of reaction was monitored by measuring the increase in absorbance at 278 nm due to the production of the tribromide ion. The bromine produced is in equilibrium with the tribromide ion (eq.15).

$$Br_2 + Br^- \longrightarrow Br_3^- \qquad \text{eq. 15}$$

The tribromide ion has a molar extinction coefficient of $3.64 \times 10^4\ M^{-1}\ cm^{-1}$ at 278nm (Libby R.D. et al 1981)

The results were expressed in terms of µmol bromine produced/min/mg and mol bromine produced/min/mol haem.

2.12.3 The Halogenation of Monochlorodimedone

The reaction mixtures contained

2.90 ml 50 mM dipotassium hydrogen phosphate buffer pH 8.0

0.05 ml 0.1M sodium chloride or sodium bromide

10 µl 15mM monochlorodimedone (dissolved in ethanol)

30 µl haem protein solution of required concentration

10 µl 50mM hydrogen peroxide to initiate the reaction.

The assays were carried out in triplicate in quartz glass cuvettes. The rate of reaction was determined by measuring the decrease in absorbance at 278 nm due to the disappearance of the monochlorodimedone.

Monochlorodimedone has a molar extinction coefficient of $1.22 \times 10^4 M^{-1} cm^{-1}$ at 278 nm (Libby R.D. et al 1981).

The results were expressed in terms of µmol monochlorodimedone utilised/min/mg and mol monochlorodimedone utilised/min/mol haem.

2.13.0 N-Demethylation Reaction of Peroxidases

The N demethylation of N, N-dimethylaniline was attempted using microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome c, (section 2.4.0) as the catalysts.

The reaction mixtures contained

2.90 ml 50 mM dipotassium hydrogen phosphate buffer pH 6.0

0.05 ml 210 mM N,N-dimethylaniline

30µl 10 mg/ml haem protein solution

10µl 40mM hydrogen peroxide to initiate the reaction.

A blank was prepared for each of the haem proteins by omitting the hydrogen peroxide. Another blank was prepared without a haem protein but with hydrogen peroxide.

The reaction mixtures were incubated at 25°C for 5 min and the reaction terminated by the addition of 0.75 ml 60% trichloroacetic acid. The reaction mixtures were twice extracted with ethyl acetate (5.0 ml) to remove any violet colouration formed during the reaction.

A 1.0 ml aliquot of the extracted aqueous phase was

incubated with 0.5 ml Nash reagent (30g ammonium acetate and 0.4 ml 2, 4-pentanedione in 50 ml) for 45 min at 25°C.

The blanks were treated in the same way and the absorbance of the samples read against their corresponding blank at 421 nm. The absorbance of the blank without a haem protein was read against distilled water. The absorbance at 421 nm is due to the formaldehyde produced. Formaldehyde has a molar extinction coefficient of 8000 M^{-1} cm^{-1} at 421 nm (Nash T. 1953).

The results were expressed in terms of µmol formaldehyde formed/min/mg and mol formaldehyde formed/min/mol haem.

2.14.0 Aromatic Hydroxylation Reactions by Peroxidases

In these experiments attempts were made to hydroxylate various aromatic compounds using microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome c (section 2.4.0) as the catalysts.

The reaction mixtures contained:

4.91 ml 50mM dipotassium hydrogen phosphate pH 8.0

30 µl 0.01M aromatic compound

30 µl 0.01M dihydroxyfumaric acid

30 µl 10 mg/ml haem protein solution.

Oxygen was bubbled into the reaction mixtures and the reactions were carried out at room temperature.

Attempts were made to hydroxylate benzene, benzylalcohol, benzaldehyde, benzoic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid, phenol and α-napthol. At various time intervals, ranging from 5 min - 4 hr, samples were removed from the reaction mixtures

and assayed. The reaction mixtures containing benzene, benzylalcohol, benzaldehyde and benzoic acid were assayed using the hydroxylated aromatic assay (Wackett L.P. and Gibson D.T. 1983). All the reaction mixtures were assayed using the o-diphenol (Arnow) assay (Waite J.H. and Tanzer M.L. 1981).

Assay to Detect Hydroxylated Aromatics

(Wackett L.P. and Gibson D.T. 1983)

In this assay the reaction of hydroxylated aromatic compounds with freshly hydrated tetrazotised o-dianisidine results in a violet reaction product. The assay contained:

0.1 ml 4.21 mM freshly hydrated tetrazotised o-dianisidine

1.0 ml reaction mixture.

The reaction was monitored by automatic scanning (1000 nm/min) of the absorption spectra from 430 - 600 nm or by the increase in absorbance at 530 nm against a blank containing distilled water.

Controls were established by the addition of 0.1 ml 4.21 mM freshly hydrated tetrazonised o-dianisidine to

i) 1.0 ml 20nM α-napthol

ii) 1.0 ml 0.01M benzylalcohol

iii) 1.0 ml reaction mixture not containing an aromatic compound.

The Colorimetric Detection of o-Diphenols: Arnow method

(Waite J.H. and Tanzer M.L. 1981).

The catecholic moiety of simple and monosubstituted o-diphenols react with nitrite to produce a bright red chromophore

in alkali.

The assay contained

0.1ml reaction mixture

0.3ml Reagent A (0.5M hydrocholoric acid)

0.3ml Reagent B (1.45M sodium nitrite, 0.41M sodium molybdate dihydrate)

0.3ml Reagent C (1.0m sodium hydroxide).

Reagents B and C were added in rapid succession. The absorbance at 500nm was determined against a blank containing 0.3ml each reagent A, B and C. The assay was proven to work using a very weak solution of noradrenaline.

The hydroxylation experiment was repeated using 30 µl undiluted benzene and 30 µl 10 mg/ml horseradish peroxidase in the reaction mixture. After one hour 0.1ml 0.1% trichloroacetic acid was added to 1.0ml reaction mixture and the resulting solution centrifuged in an Eppendorf microfuge for 5 minutes to remove any precipitate. The presence of hydroxylated aromatics was tested for using the two assays as before.

2.14.1 The Polymerisation of Hydroxylated Aromatic Compounds

The results from section 2.14.0 suggested that a polymerisation process may be occurring. This was investigated as follows.

Two reaction mixtures were set up

i) 9.82ml 40mM dipotassium hydrogen phosphate buffer pH 6.0

60 µl 1.0M α-napthol

10 µl 0.01M dihydroxyfumaric acid

60 µl 10 mg/ml trypsin digested cytochrome c.

ii) as above with 60 µl 40mM hydrogen peroxide in lieu of dihydroxyfumaric acid.

The reaction mixtures were incubated at room temperature. Oxygen was bubbled into the reaction mixture containing dihydroxyfumaric acid but not into the reaction mixture containing hydrogen peroxide.

1.0ml samples were removed at time 0 min and at 10 min intervals for 80 min. The reaction was followed by measuring the maximum absorbance of 1.0 ml reaction mixture and 0.1 ml freshly hydrated tetrazotised o-dianisidine at 530 nm. It was assumed that if the ∝-napthol was being polymerised in the reaction mixture a decrease in the absorbance at 530nm would be observed. The o-diphenol assay was also performed.

2.15.0 The Immobilisation of Peroxidases

Horseradish peroxidase, pepsin digested cytochrome c and trypsin digested cytochrome c (section 2.4.0) were immobilised on three immobilisation supports.

The supports chosen for the experiment were

i) controlled pore glass beads (mesh size 80-120)

ii) Macrosorb K (GA), a macroporous kiesselguhr granule surface coated with gluteraldehyde

iii) UOP IPS-100, a composite of a polymer - gluteraldehyde complex bound to an inert alumina carrier.

The Macrosorb K (GA) support was supplied as a slurry in water. The support was washed five times with 50 mM dipotassium hydrogen phosphate buffer pH 8.0 and as much excess fluid removed as possible.

5mg/ml solutions of each haem protein were prepared and the number of haem groups present in each estimated by measuring their absorbance in the Soret region (see section 2.5.0).

1.0ml aliquots of each haem protein were added to 50 mg of each support in small glass vials. The immobilisation was carried out for approximately 20 hours at 4°C. The vials were placed on a rocking table to ensure an even coating of the support particles.

The following day the excess solutions were removed from the supports and the number of haems present in the solutions estimated as before. The supports were washed at least three times with 50mM dipotassium hydrogen phosphate buffer pH 8.0 to remove unbound haem protein and the peroxidase activity of the immobilised haem proteins was assayed as follows:

10 mg immobilised enzyme

5.80 ml 50mM dipotassium hydrogen phosphate buffer pH 8.0

0.1ml 20mM guaiacol

20 µl 40mM hydrogen peroxide to initiate the reaction.

The reaction mixtures were shaken by hand for one minute and 1.0ml removed. The absorbance at 470 nm due to the production of tetraguaiacol was determined against a blank of phosphate buffer and guaiacol.

An estimation of the number of haems bound to each support was made by subtracting the number of haems in the solution after immobilisation from the number of haems in the solution before immobilisation.

3.0.0 RESULTS

3.1.0 The Guaiacol Assay for Peroxidase Activity

Table 2 A Comparison of the Peroxidase Activity of Six Haem Proteins Under the Same Conditions

HAEM PROTEIN	AVERAGE ACTIVITY IN	
	µmol/min/mg	mol/min/mol haem
mp-11	2.296	59.26
HRP	15.864	1624.71
cyt. c	0.513×10^{-3}	6.36×10^{-3}
Hb	9.361×10^{-3}	0.12
P	1.454	21.89
T	0.367	41.41

Abbreviations

mp-11 = microperoxidase mp-11

HRP = horseradish peroxidase

cyt.c = cytochrome c

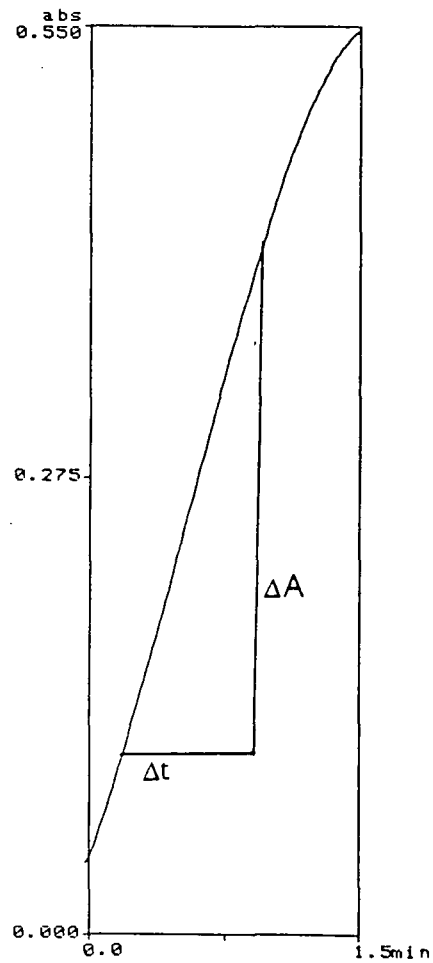
Hb = haemoglobin

P = pepsin digested cytochrome c

T = trypsin digested cytochrome c

Calculation

Fig.4 The Increase in Absorbance at 470nm Due to the Production of Tetraguaiacol



Change in absorbance $= \Delta A / \Delta t$

change in time

$\frac{\Delta A / \Delta t}{\varepsilon}$ Molar/min = C

C x volume of assay = moles/min

$\frac{\text{moles/min}}{\text{concentration of peroxidase}}$ = moles/min/mg

where ε = molar extinction coefficient = $2.66 \times 10^{4} M^{-1} cm^{-1}$

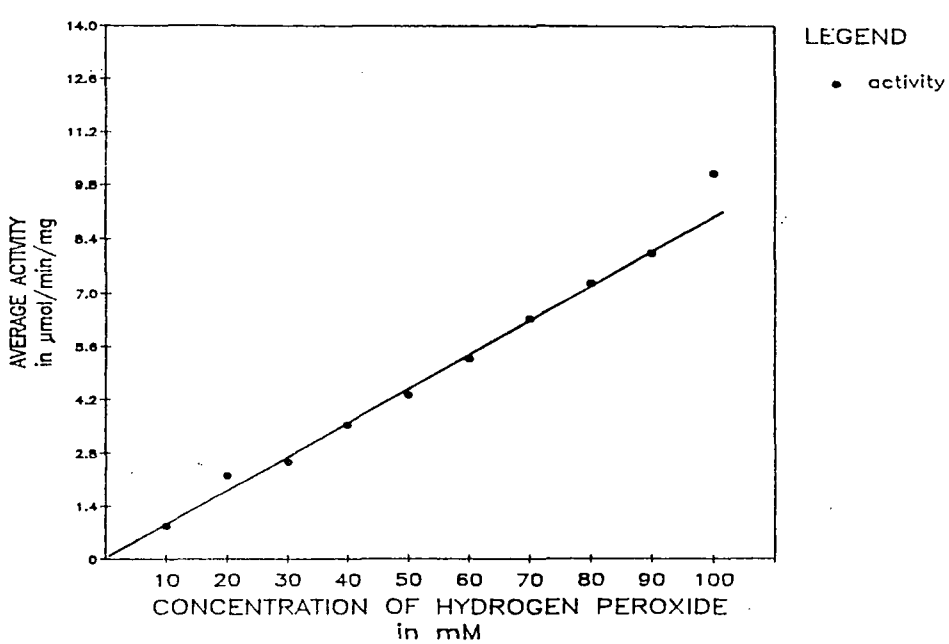
volume of assay = 3×10^{-6} l.

3.1.1 The effect of Substrate Concentration on the Guaiacol Assay

Table 3

CONCENTRATION OF HYDROGEN PEROXIDE (mM)	AVERAGE ACTIVITY (µmol/min/mg)
10	0.862
20	2.196
30	2.553
40	3.513
50	4.310
60	5.262
70	6.297
80	7.230
90	8.022
100	10.066

Fig. 5 Graph of Concentration of Hydrogen Peroxide vs. Average Activity.

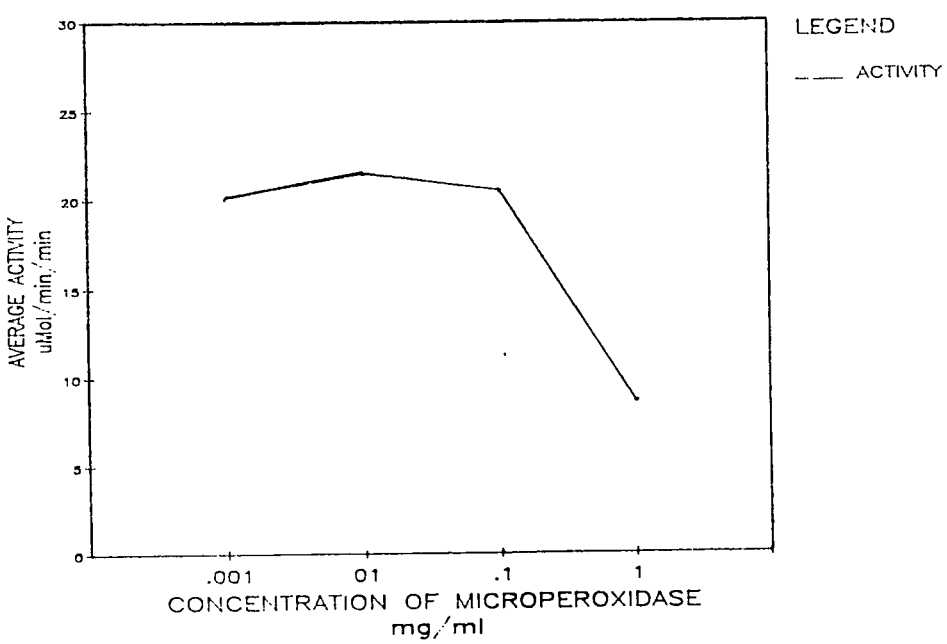


3.1.2 The Effect of Peroxidase Concentration on the Guaiacol Assay

Table 4

CONCENTRATION OF MICROPEROXIDASE mp-11	AVERAGE ACTIVITY (µmol/min/mg)
50 µl 0.001 mg/ml	20.12
30 µl 0.01 mg/ml	21.45
10 µl 0.1 mg/ml	20.49
10 µl 1.0 mg/ml	8.60

Fig. 6 The Effect of Peroxidase Concentration on the Guaiacol Assay



The results are expressed in terms of µmol tetraguaiacol formed/min/mg microperoxidase mp-11.

3.2.0 The Proteolytic Digestion of Haemoglobin and Cytochrome c

Table 5 The Proteolytic Digestion of Haemoglobin

PROTEOLYTIC ENZYME	AVERAGE ACTIVITY (µmol/min/mg) at TIME (hr)		
	1	2	3
Pepsin	0.016	0.016	0.016
Protease type XXVII	0.015	0.013	0.012
Trypsin	0.015	0.015	0.015
DPCC - trypsin	0.017	0.017	0.016

There was no peroxidase activity remaining after four hours of digestion.

Table 6 The Pepsin Digestion of Haemoglobin

TIME OF DIGESTION (min)	ACTIVITY (µmol tetraguaiacol/min/mg Hb)
5	0.017
10	0.017
15	0.016
20	0.017
25	0.015
30	0.018
35	0.012
40	0.012
45	0.014
50	0.012
55	0.014
60	0.011
65	0.012
70	0.013
75	0.012

Fig. 7 Graph of Activity vs. Time of Digestion

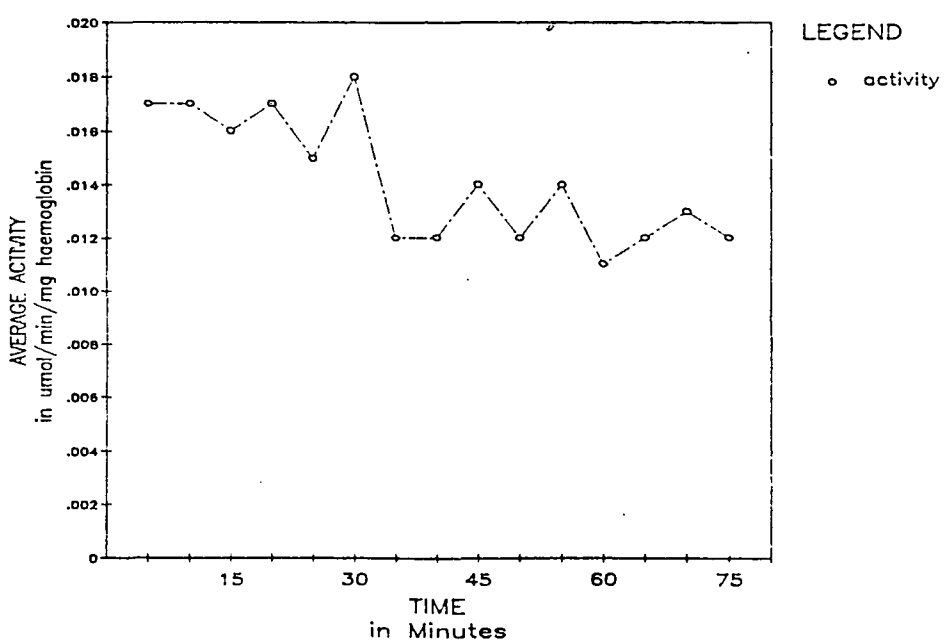


Fig.8 The Pepsin Digestion of Cytochrome c

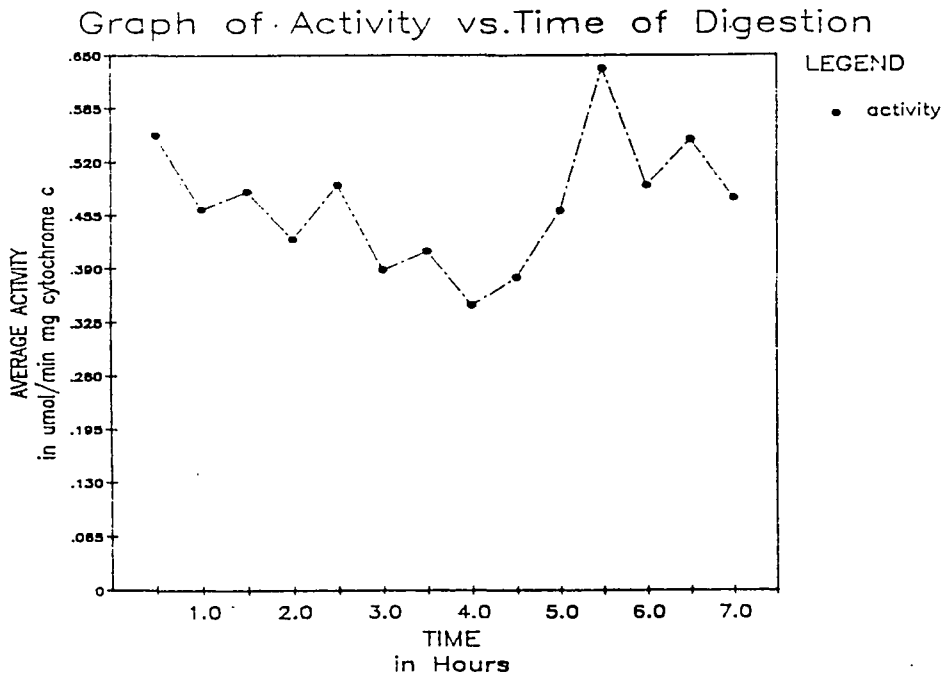


Fig.9 The Protease type XXVII Digestion of Cytochrome c

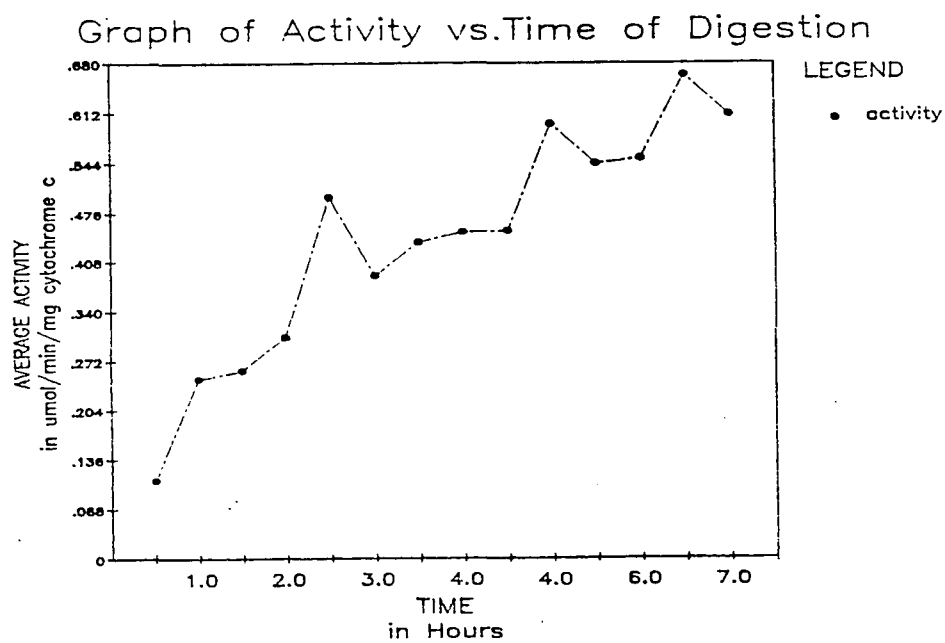


Fig. 10 The Trypsin Digestion of Cytochrome c

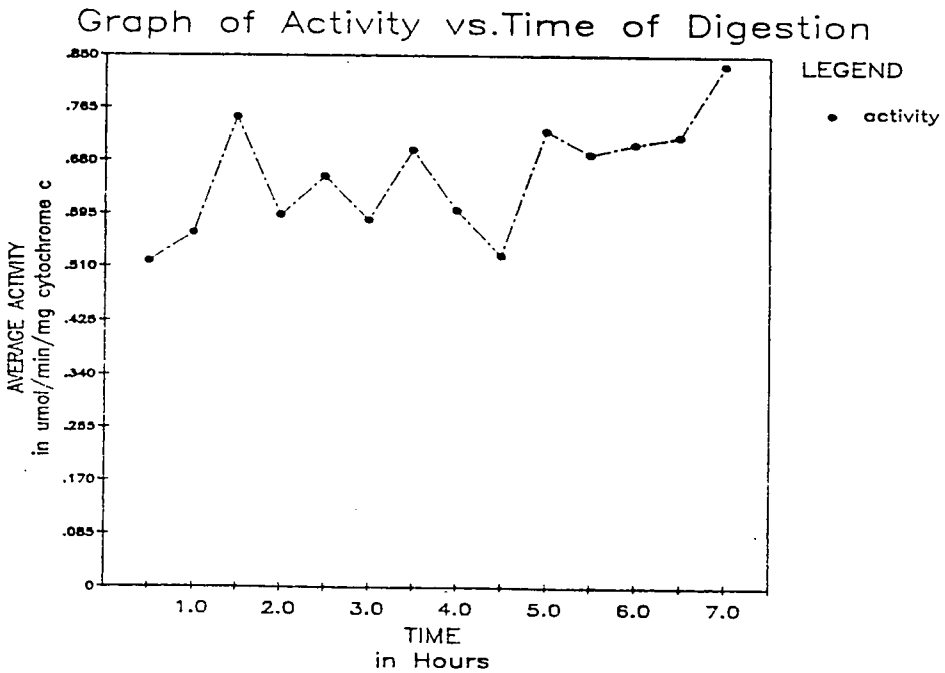
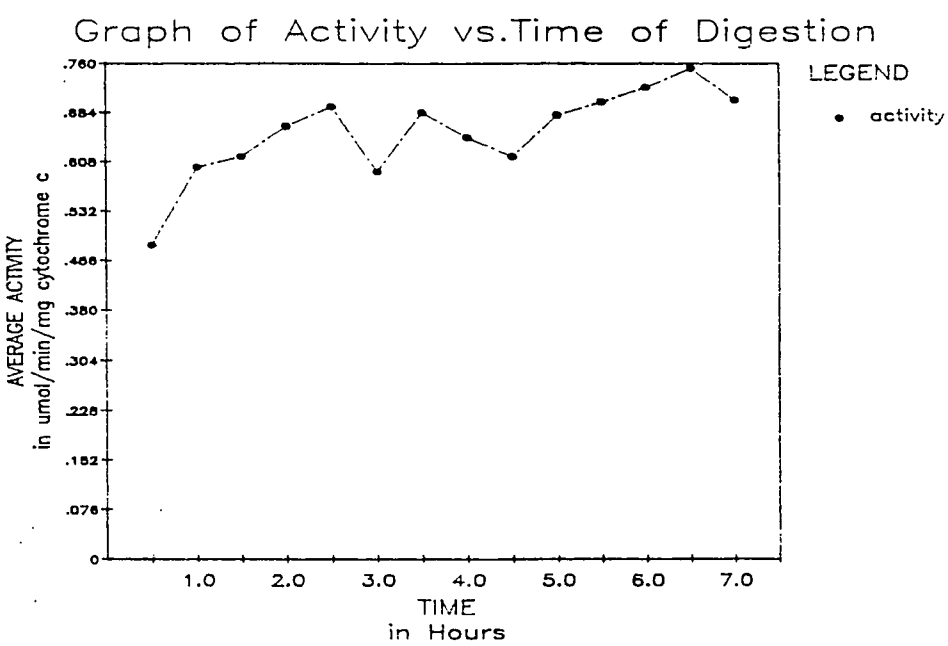


Fig.11 The DPCC - Trypsin Digestion of Cytochrome c



3.3.0 The Gel Filtration of Pepsin Digested Cytochrome c

The Sephadex G15 (0.5 x 10 cm) and Sephadex G25 (0.5 x 10 cm) columns did not separate a mixture of digested and undigested cytochrome c. Slight separation was observed on Sephadex G50 (1.0 x 30 cm) column, better separation was obtained on the Sephadex G50 (1.5 x 75 cm) column (Table 7, Fig. 12). The separation was improved using a dissociating buffer. (Table 8, Fig. 13).

Table 7 Gel Filtration of Digested and Undigested Cytochrome c on Sephadex G50 using a Non-Dissociating Buffer

ELUTION TIME (min)	POSITION OF SORET PEAK (nm)	ABSORBANCE OF SORET PEAK
58	407	0.029
61	408	0.283
64	408	0.504
67	408	0.499
70	407	0.199
73	406	0.123
76	404	0.156
79	403	0.141
82	402	0.103
85	400	0.062
88	399	0.041
91	400	0.022
94	400	0.008
97	401	0.001

Fig.12 Graph of Absorbance in Soret Region vs. Elution Time

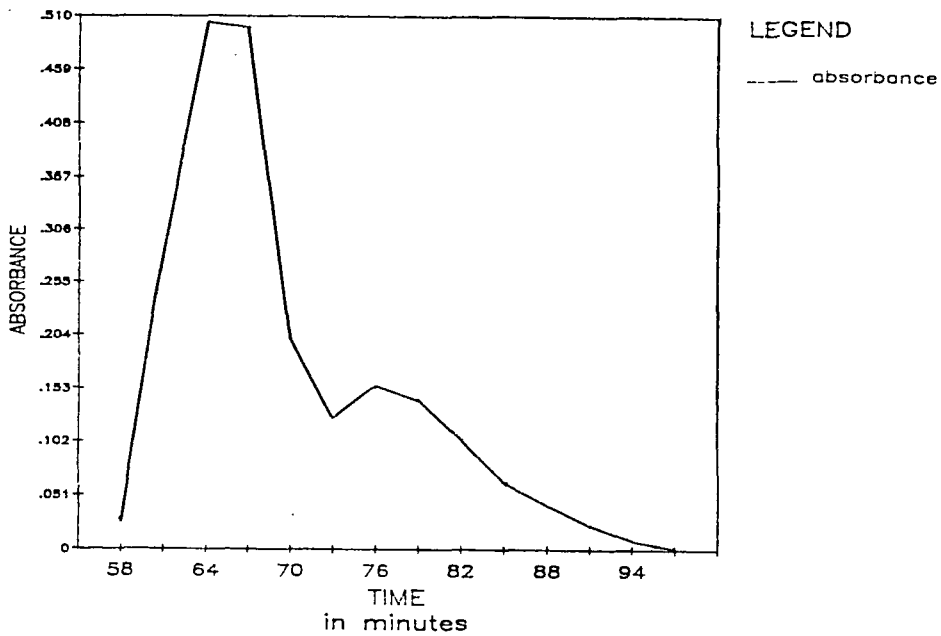


Table 8 Gel Filtration of Digested and Undigested Cytochrome C on Sephadex G50 using a Dissociating Buffer

ELUTION TIME (min)	POSITION OF SORET PEAK (nm)	ABSORBANCE IN SORET REGION
46	407	0.022
48	408	0.077
50	408	0.147
52	408	0.179
54	408	0.156
56	408	0.114
58	408	0.074
60	400	0.068
62	399	0.117
64	399	0.111
66	399	0.158
68	399	0.155
70	399	0.139
72	398	0.110
74	398	0.084
76	398	0.056
78	398	0.035
80	398	0.025

Fig.13 Graph of Absorbance in Soret Region vs. Time of Elution

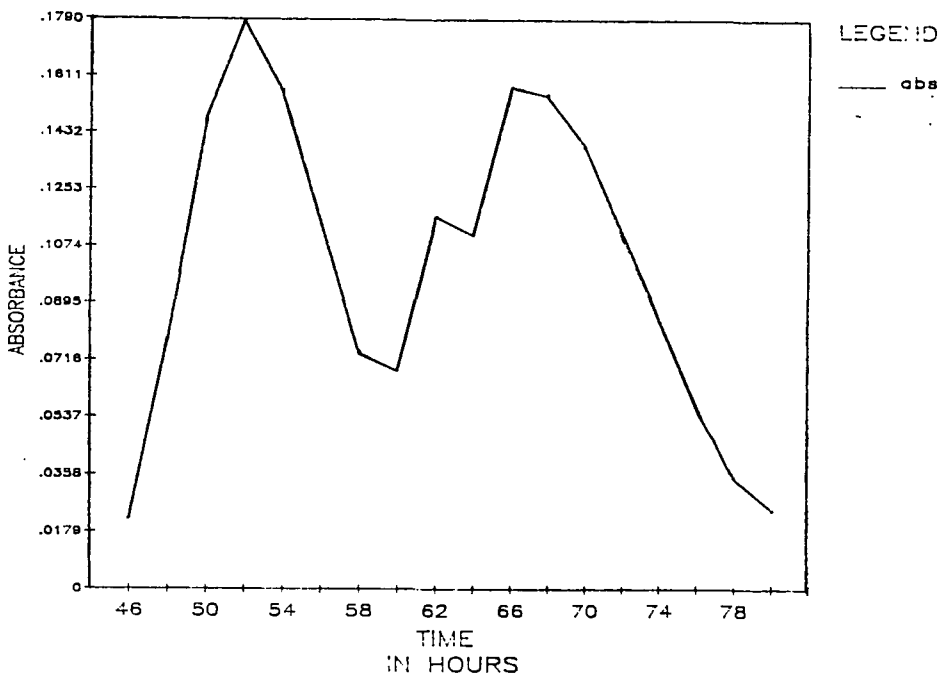


Table 9 The Peroxidase Activity of the Eluted Fractions

ELUTION TIME (min)	ACTIVITY OF ELUTED FRACTION (mol/min/mol haem)
62	70.06
64	11.50
66	53.82
68	53.20
70	51.35
72	45.70

Activity of the digest not passed through the column = 28.39 mol tetraguaiacol formed/min/mol haem.

3.3.1 The Gel Filtration of Pepsin Digested Cytochrome c After Different Times of Digestion

The results are too numerous to show here and may be found in my Practical Book No. 1.

There was no separation of pepsin digested fragments on the Sephadex G50 (1.5 x 75 cm) column using a dissociating column. The peroxidase assay however showed that most of the activity was located in fractions eluted between approximately 60-80 min. The first fraction to be eluted with a positive absorbance in the soret region was typically after approximately 50 min.

As the fractions were eluted, the position of the Soret bond shifted towards the blue and the absorbance at 280 nm decreased to nothing.

3.3.2 The Gel Filtration of Pepsin Digested Cytochrome c on PD-10 Columns

Table 10 The Specific Activity and Total Activity of Fractions Eluted using a Non-Dissociating Buffer

FRACTION NUMBER	SPECIFIC ACTIVITY (mol/min/mol haem)	TOTAL ACTIVITY (mol/min)
1	40.39	0.466
2	36.28	2.200
3	71.26	0.361
4	99.37	0.125
5	121.33	0.055

Total activity loaded = 2.860 mol/min

Total activity eluted = 3.207 mol/min

Fig. 14 Graph of Specific Activity and Total Activity Against Fraction Number of Fractions Eluted From a PD-10 Column with a Non-Dissociating Buffer

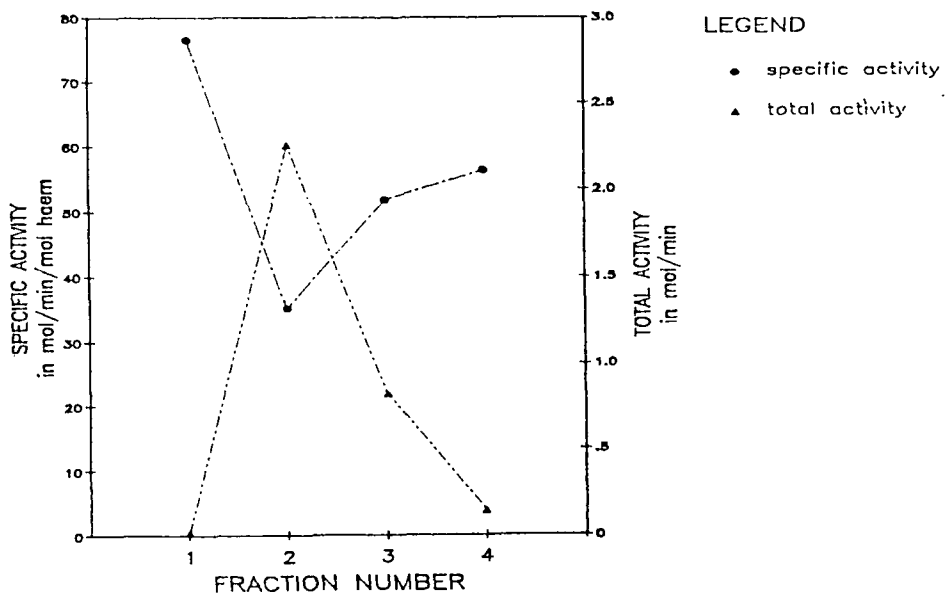


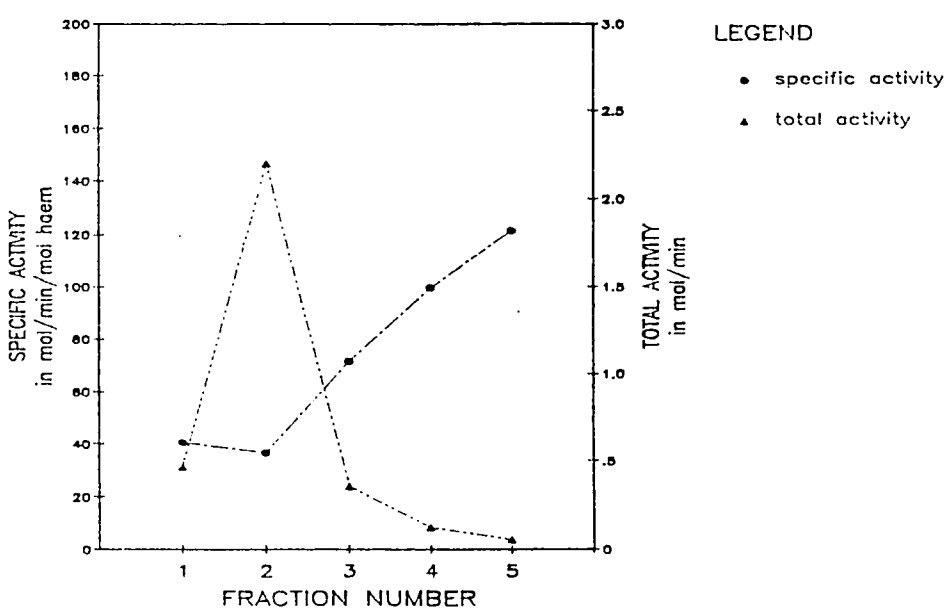
Table 11 The Specific Activity and Total Activity of Fractions Eluted Using a Dissociating Buffer

FRACTION NUMBER	SPECIFIC ACTIVITY (mol/min/mol haem)	TOTAL ACTIVITY (mol/min)
1	76.49	0.186
2	35.05	2.260
3	51.72	0.823
4	56.24	0.146

Total activity loaded = 2.860 mol/min

Total activity eluted = 3.415 mol/min

Fig.15 Graph of Specific Activity and Total Activity Against Fraction Number (using a Dissociating Buffer to Elute the Fractions)



3.3.3 The Effect of Hydrogen Peroxide and Sodium Dithionite on Pepsin Digested Cytocrhome c

Table 12 The Effect of Hydrogen Peroxide on the Absorbance in the Soret Region

TIME (min)	POSITION OF SORET PEAK (nm)	ABSORBANCE OF SORET PEAK
0	407	0.298
5	406	0.220
10	406	0.195
15	406	0.179
20	406	0.167
25	406	0.158
30	406	0.152
35	406	0.145
40	406	0.144
45	406	0.140
50	406	0.137
55	406	0.134
60	406	0.132
65	406	0.130
70	406	0.128
75	406	0.126
80	406	0.125
85	406	0.124

Fig.16 The Effect of Hydrogen Peroxide on Pepsin Digested Cytochrome C

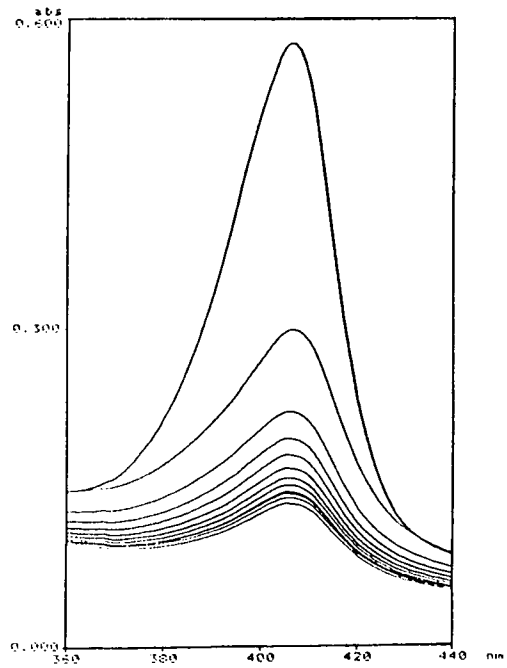
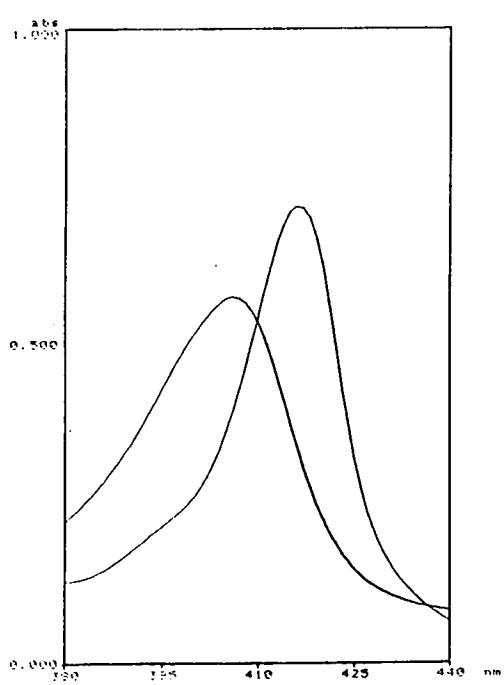


Fig.17 The Effect of Sodium Dithionite on Pepsin Digested Cytochrome c



3.4.0 The Preparation of Microperoxidases for Further Experiments

Lyophilysation of the pepsin digest of cytochrome c yielded a dark reddish-pink, fluffy product. The lyophilised trypsin digest of cytochrome c was a paler, pinker, more powdery product.

It was necessary to repeat the digests, and although the conditions were kept as constant as possible, the products were very different in their activities.

3.4.1 The Effect of Lyophilisation on Peroxidase Activity

Following lyophilisation of pepsin digested cytochrome c, the activity decreased from approximately 40 mol tetraguaiacol formed/min/mol haem to approximately 3 mol tetraguaiacol formed/min/mol haem.

A similar reduction was noted for the trypsin digested cytochrome c.

3.5.0 An Estimation of the Number of Haems

Table 13 The Absorbance of the Haem Proteins in the Soret Region

HAEM PROTEIN	CONCENTRATION (mg/ml)	SORET PEAK (nm)	ABSORBANCE OF SORET PEAK
mp-11	0.01	398.4	0.130
HRP	0.50	402.6	0.454
cyt. c	0.10	411.0	0.730
Hb	0.10	406.8	0.755
P	0.10	406.0	0.532
T	0.10	396.0	0.087

(See Table 2 for abbreviations)

Figs. 18 to 24 show absorption spectra of the six haem proteins. The spectra however do not correspond to these results, but they do show the appearance of the spectra and the positions of the Soret peak.

Fig. 18 Absorption Spectra of Microperoxidase mp-11

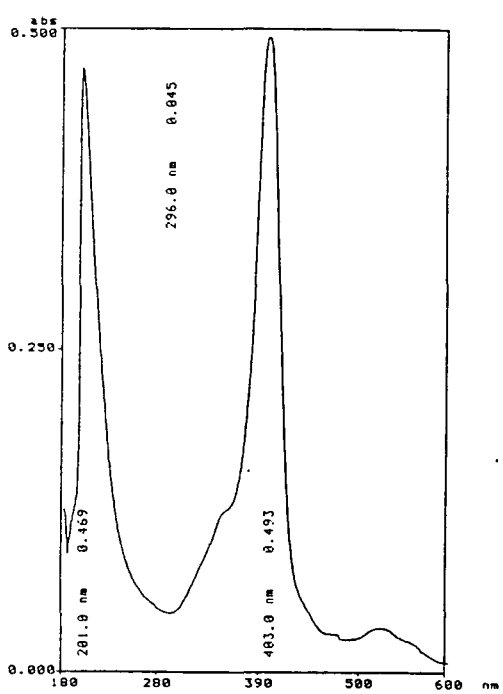


Fig. 19 Absorption Spectra of Horseradish peroxidase

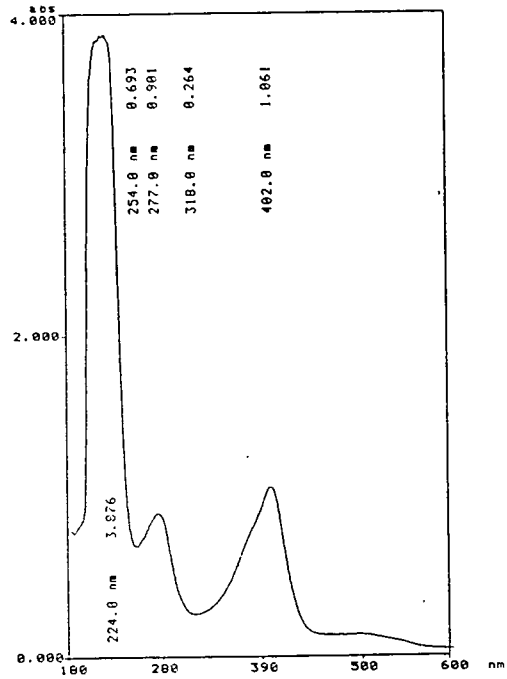


Fig. 20 Absorption spectra of Cytochrome c

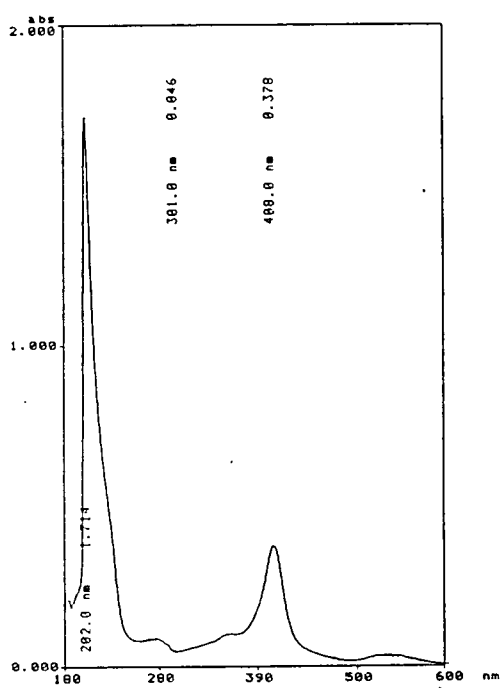


Fig. 21 Absorption spectra of Haemoglobin

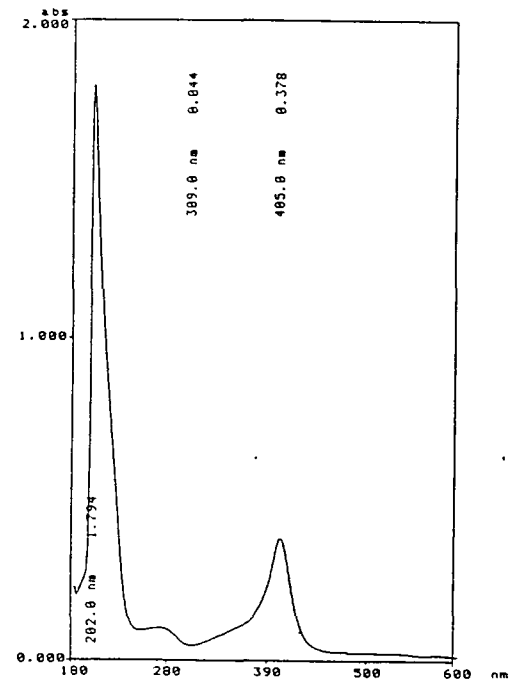


Fig. 22 Absorption spectra of Pepsin Digested Cytochrome c

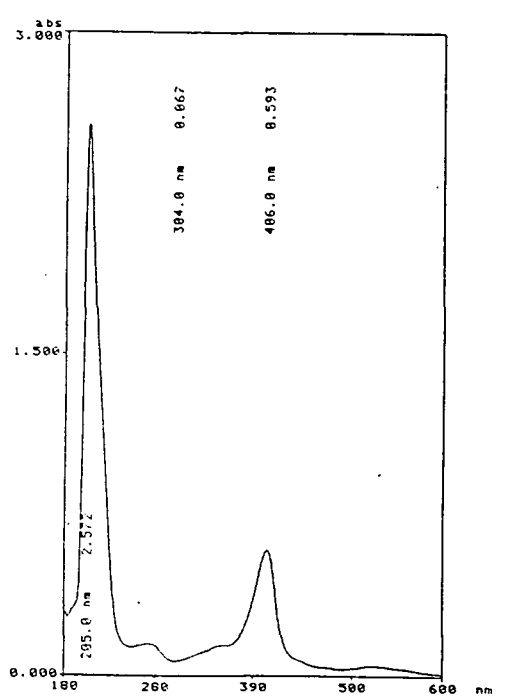
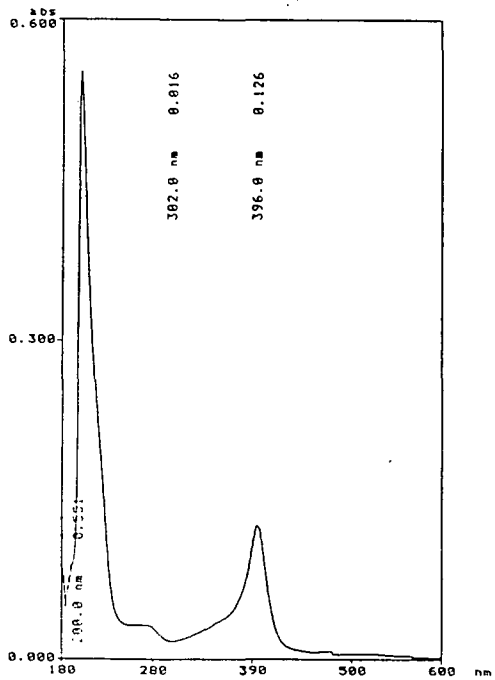


Fig. 23 Absorption spectra of Trypsin Digested Cytochrome c



Calculation

Cytochrome c possesses one haem group per molecule (Paul K.G. 1955).

The molecular weight of cytochrome c type III = 12,384

0.1 mg cytochrome c/ml = 0.1g/l

$$\frac{0.1}{12,384} = 8,075 \times 10^{-6} \text{ M}$$

The number of haems in 0.1g therefore =

$$8.076 \times 10^{-6} \times 6.022 \times 10^{23}$$

$$= 4.86 \times 10^{18} \text{ haems/0.1g}$$

$$= 4.86 \times 10^{15} \text{ haems/mg cytochrome c}$$

The haem extinction coefficient was then determined

Absorbance $= \varepsilon . c . l$ where

ε = extinction coefficient

c = concentration

l = path length

$0.755 = \varepsilon \times 8.075 \times 10^{-6} \times 1.0$

$\varepsilon = 9300\ ^{-1}\ cm^{-1}$

To calculate the number of haems/mg in the other haem proteins the following calculation was used.

x mg/ml scanned is equivalent to xg/l

$c = \frac{A}{\varepsilon} = y \times 10^{-6}$ M

$y \times 10^{-6} \times 6.022 \times 10^{23}$ = No. of haems/xg

e.g. for haemoglobin 0.1 mg/ml was scanned which is equivalent to 0.1g/l

$c = \frac{A}{\varepsilon}$

A = 0.730 therefore $c = 7.85 \times 10^{-6}$ M

$7.85 \times 10^{-6} \times 6.022 \times 10^{23} = 4.73 \times 10^{18}$ haems/0.1g

$= 4.73 \times 10^{16}$ haems/mg

Table 14 The Number of Haems/mg of Six Haem Proteins

HAEM PROTEIN	NUMBER OF HAEMS/mg
mp-11	8.43×10^{16}
HRP	5.88×10^{15}
cyt.c	4.86×10^{16}
Hb	4.73×10^{16}
P	3.42×10^{16}
T	5.60×10^{15}

(See Table 2 for abbreviations)

3.6.0 The pH Profiles of Microperoxidase mp-11, Horseradish Peroxidase, Pepsin Digested Cytochrome c, and Trypsin Digested Cytochrome c

The peroxidase activity of the above haem proteins were assayed in buffers of different pH.

Table 15 The Peroxidase Activity of Microperoxidase mp-11 in Buffers of Different pH

pH (and Buffer)	AVERAGE ACTIVITY	
	(μmol/min/mg)	(mol/min/mol haem)
2.6 (citrate)	0.017	0.12
3.0 (citrate)	0.026	0.19
3.4 (citrate)	0.038	0.27
3.8 (citrate)	0.056	0.39
4.2 (citrate)	0.084	0.60
4.6 (citrate)	0.112	0.80
5.0 (citrate)	0.218	1.56
5.4 (citrate)	0.291	2.08
5.8 (citrate)	0.389	2.78
6.2 (citrate)	0.676	4.83
6.6 (citrate)	1.252	8.94
7.0 (citrate)	2.078	14.84
7.4 (citrate)	5.933	42.38
7.4 (tris)	12.914	92.25
7.8 (tris)	10.064	76.01
8.2 (tris)	13.421	95.87
8.6 (tris)	17.932	128.10
8.6 (glycine)	2.109	15.07
9.0 (glycine)	1.759	12.57
9.4 (glycine)	1.406	10.04
9.8 (glycine)	1.170	8.36
10.4 (glycine)	0.956	6.83
11.0 (phosphate)	7.887	56.34
11.4 (phosphate)	3.462	24.73
11.8 (phosphate)	1.459	10.42

Fig. 24 The pH Profile of Microperoxidase mp-11

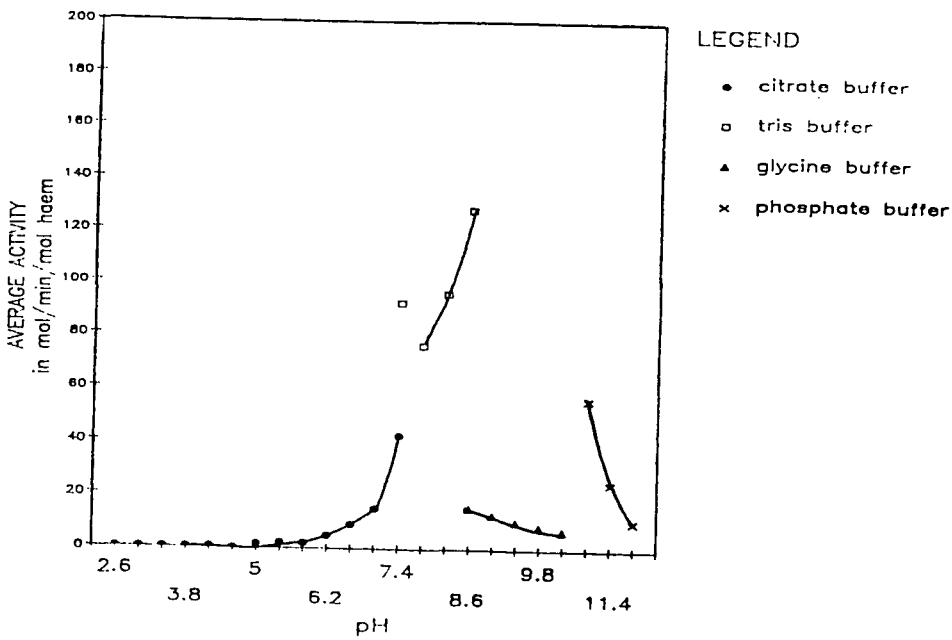


Table 16 Peroxidase Activity of Microperoxidase mp-11 in Bis Tris Buffers

pH	AVERAGE ACTIVITY	
	(μmol/min/mg)	(mol/min/mol haem)
5.8	0.441	3.15
6.0	0.459	3.28
6.5	0.725	5.18
7.0	1.783	12.74

Table 17 Peroxidase Activity of Microperoxidase mp-11 in Tricine Buffers

pH	AVERAGE ACTIVITY	
	(μmol/min/mg)	(mol/min/mol haem)
7.6	4.865	34.74
8.0	4.710	33.65
8.5	9.209	65.79

Table 18 The Peroxidase Activity of Microperoxidase mph-11 in Bis Tris Propane Buffers

pH	AVERAGE ACTIVITY	
	(μmol/min/mg)	(mol/min/mol haem)
6.5	9.749	69.64
7.0	15.113	107.96
7.5	20.414	145.83
8.0	25.263	180.47
8.5	25.489	182.08
9.0	25.902	185.03
9.5	22.105	157.91

Fig.25 The pH Profile of Microperoxidase mp-11 (in Bis Tris Propane Buffer)

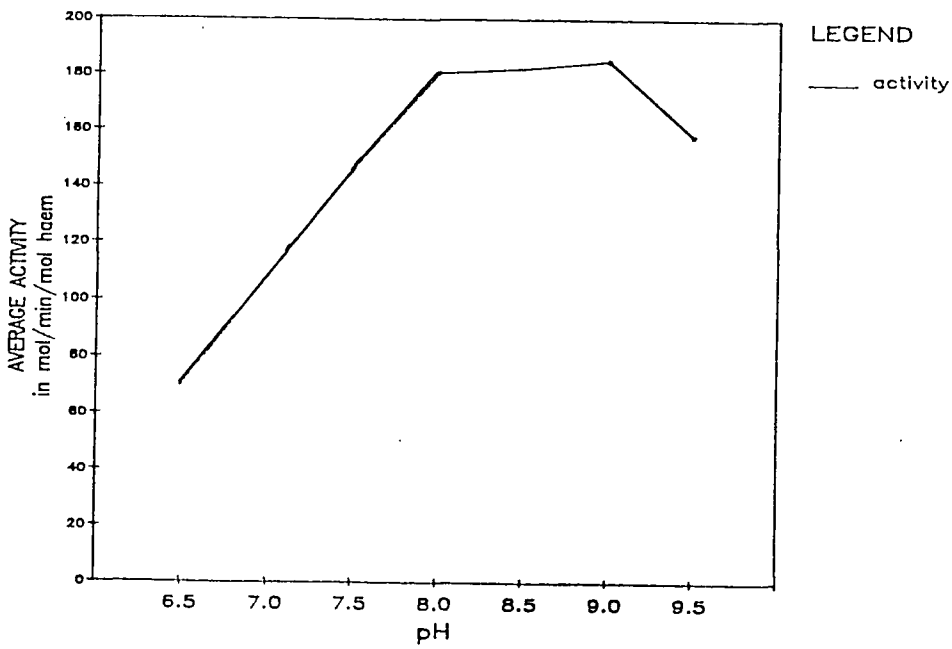


Table 19 The Peroxidase Activity of Horseradish Peroxidase in Buffers of Different pH

pH (and Buffer)	AVERAGE ACTIVITY	
	(μmol/min/mg)	(mol/min/mol haem)
2.6 (citrate)	2.080	213.02
3.0 (citrate)	1.973	202.07
3.4 (citrate)	2.857	292.60
3.8 (citrate)	4.373	447.90
4.2 (citrate)	6.140	628.80
4.6 (citrate)	7.443	762.30
5.0 (citrate)	6.457	661.30
5.4 (citrate)	6.980	714.90
5.8 (citrate)	7.281	745.70
6.2 (citrate)	7.507	768.80
6.6 (citrate)	6.899	706.60
7.0 (citrate)	6.479	663.60
7.4 (citrate)	6.222	637.20
7.4 (tris)	7.951	814.30
7.8 (tris)	8.509	871.50
8.2 (tris)	6.479	663.60
8.6 (tris)	5.401	553.10
8.6 (glycine)	5.132	525.60
9.0 (glycine)	4.749	486.40
9.4 (glycine)	1.629	166.80
9.8 (glycine)	0.326	33.39
10.4 (glycine)	0.200	20.48
11.0 (phosphate)	-	-
11.4 (phosphate)	-	-
11.8 (phosphate)	-	-

Fig.2b The pH Profile of Horseradish Peroxidase

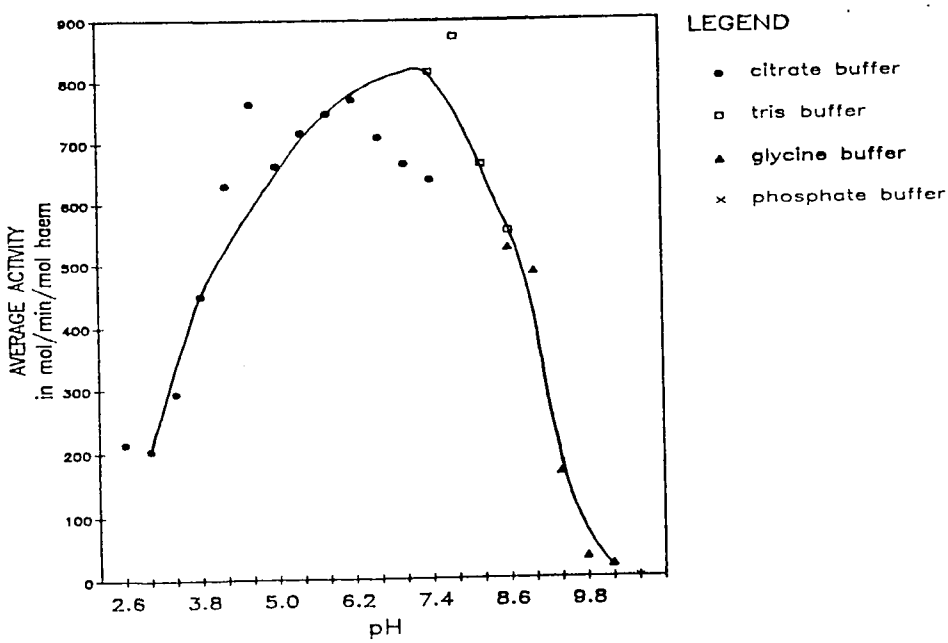


Table 20 The Peroxidase Activity of Pepsin Digested Cytochrome c in Buffers of Different pH

pH	AVERAGE ACTIVITY	
	(μmol/min/mg)	(mol/min/mol haem)
4.0	8.321×10^{-3}	0.15
4.5	0.0132	0.23
5.0	0.0127	0.22
5.5	0.0252	0.44
6.0	0.0383	0.67
6.5	0.0952	1.67
7.0	0.638	11.20
7.5	1.553	27.26
8.0	2.877	50.50
8.5	5.376	94.36
9.0	8.571	150.44
9.5	8.471	148.68
10.0	8.120	142.52
10.5	6.516	113.31
11.0	3.722	65.33
11.5	2.263	39.72

Fig.27 The pH Profile of Pepsin Digested Cytochrome C

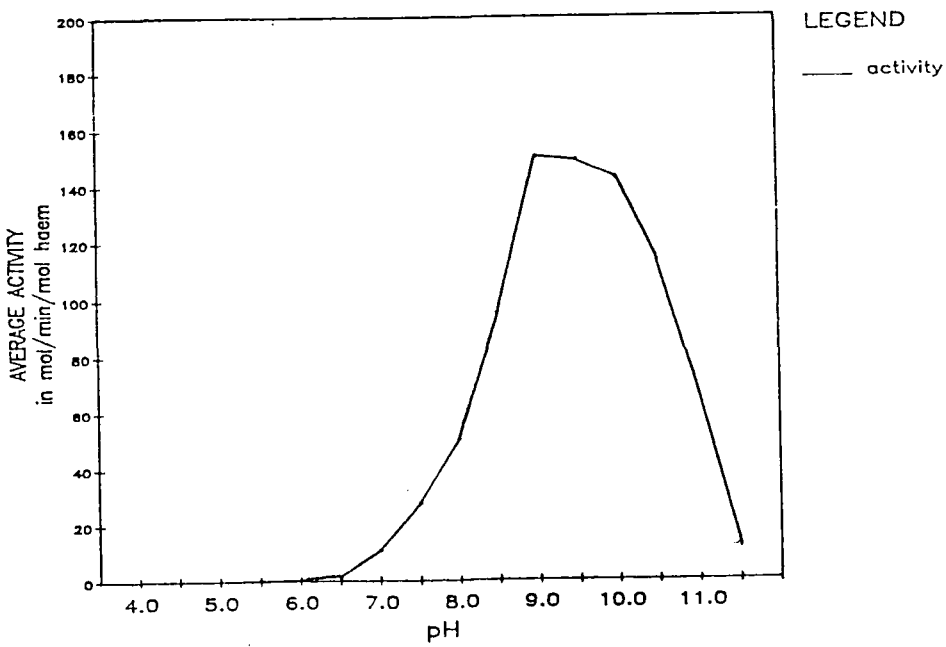
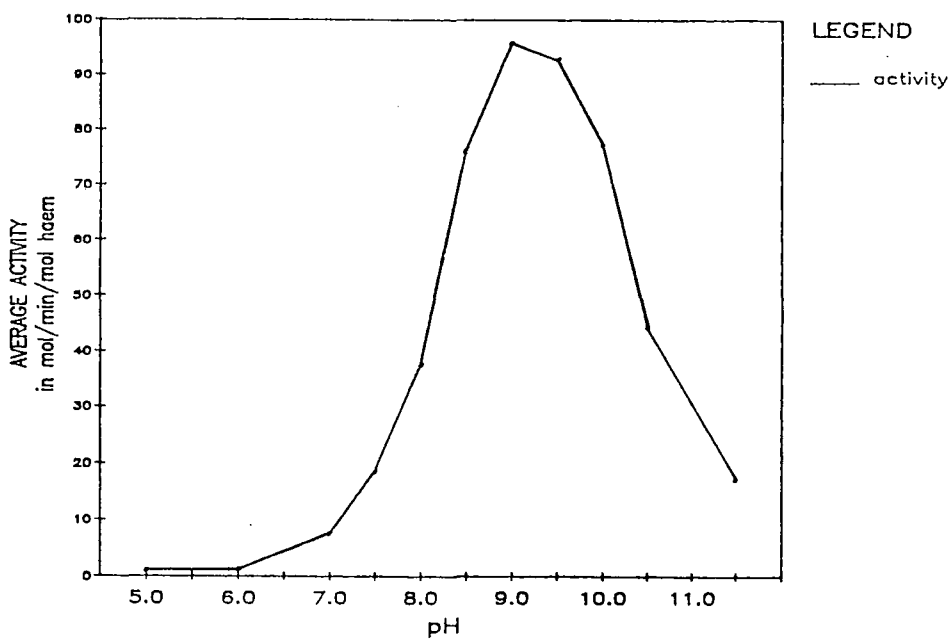


Table 21 The Peroxidase Activity of Trypsin Digested Cytochrome c in Buffers of Different pH

pH	AVERAGE ACTIVITY	
	(μmol/min/mg)	(mol/min/mol haem)
5.0	9.624×10^{-3}	1.04
5.5	0.010	1.08
6.0	0.010	1.08
6.5		
7.0	0.070	7.56
7.5	0.173	18.60
8.0	0.348	37.42
8.5	0.709	76.24
9.0	0.892	95.92
9.5	0.862	92.70
10.0	0.718	77.21
10.5	0.417	44.84
11.0	0.251	76.99
11.5	0.161	17.31

(NB: The peroxidase activity was not measured at pH 6.5)

Fig.28 The pH Profile of Trypsin Digested Cytochrome c



3.7.0 The pH Stability of Microperoxidase mp-11 and Horseradish Peroxidase

Table 22 The pH Stability of Microperoxidase mp-11

pH	AVERAGE ACTIVITY (µmol/min/mg) AFTER						
	0 hr	4 hr	6 hr	27 hr	48 hr	1 wk	1 mth
3.0	0.716	1.115	0.617	1.070	0.907	NS	NS
4.0	0.919	1.466	0.890	1.183	1.042	1.010	1.010
5.0	0.848	1.045	0.812	0.561	0.363	0.265	0.267
6.0	0.775	0.961	0.698	0.470	0.289	0.231	0.291
7.0	1.822	1.985	1.641	1.303	0.931	0.786	0.691
8.0	1.974	2.042	1.663	1.275	0.782	0.596	0.616
9.0	2.515	2.656	2.521	2.064	1.794	1.895	1.822
10.0	2.594	3.243	2.667	2.555	2.312	2.199	2.527
11.0	2.132	2.869	2.183	0.921	1.495	1.432	1.686

NS = No sample left to assay.

Table 23 The pH Stability of Horseradish Peroxidase

pH	AVERAGE ACTIVITY (µmol/min/mg) AFTER						
	0 hr	3 hr	5 hr	24 hr	48 hr	1 wk	1 mth
3.0	11.958	5.611	5.367	0.957	0.539	0.000	0.000
4.0	12.923	14.117	14.586	12.969	12.820	7.641	0.000
5.0	12.754	11.363	13.892	9.634	11.335	4.211	NS
6.0	14.079	12.171	14.126	14.549	12.491	7.040	9.126
7.0	8.652	6.391	9.013	5.874	8.261	6.965	5.376
8.0	8.844	7.077	8.891	7.923	7.726	6.655	3.271
9.0	13.496	10.414	11.974	12.105	12.782	11.053	9.596
10.0	9.267	10.968	13.872	14.098	13.478	12.012	7.961
11.0	12.181	9.107	11.867	12.068	11.400	10.056	7.134

NS No sample left to assay.

3.8.0 The Temperature Profiles of Microperoxidase mp-11, Horseradish Peroxidase, Pepsin Digested Cytochrome c and Trypsin Digested Cytochrome c

The peroxidase activity of the above haem proteins was measured at different temperatures. The results are expressed in terms of μmol/min/mg and mol/min/mol haem.

Table 24 Peroxidase Activity of Microperoxidase mp-11 at Different Temperatures

TEMPERATURE (°C)	AVERAGE ACTIVITY (μmol/min/mg)	(mol/min/mol haems)
11	16.085	114.90
18	17.556	125.41
22	16.241	116.02
26	17.970	128.37
30	19.286	137.77
37	23.515	167.98
40	22.632	161.67
45	21.128	150.93
50	19.549	139.65
55	19.587	139.92
57	18.271	130.52

Fig.29 Temperature Profile of Microperoxidase mp-11

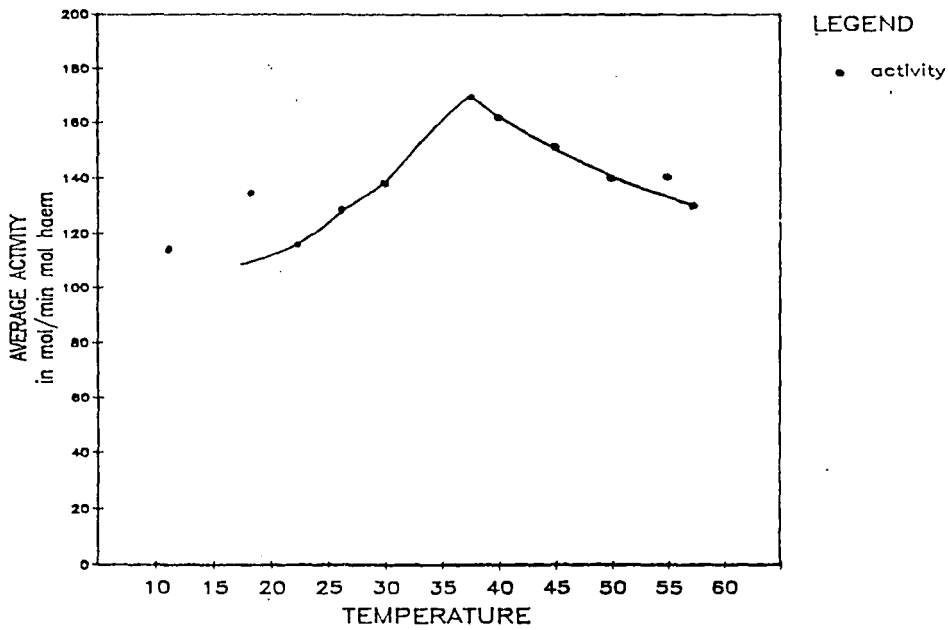


Table 25 The Peroxidase Activity of Horseradish Peroxidase at Different Temperatures

TEMPERATURE (°C)	AVERAGE ACTIVITY (μmol/min/mg)	(mol/min/mol haems)
12	5.827	596.77
15	6.128	627.60
17	7.011	718.03
20	7.989	818.19
25	10.708	1096.66
30	13.384	1370.72
35	15.664	1604.22
40	17.594	1801.88
45	20.733	2123.36
50	22.995	2355.03
55	25.501	2611.68
60	28.246	2892.81

Fig.30 Temperature Profile of Horseradish Peroxidase

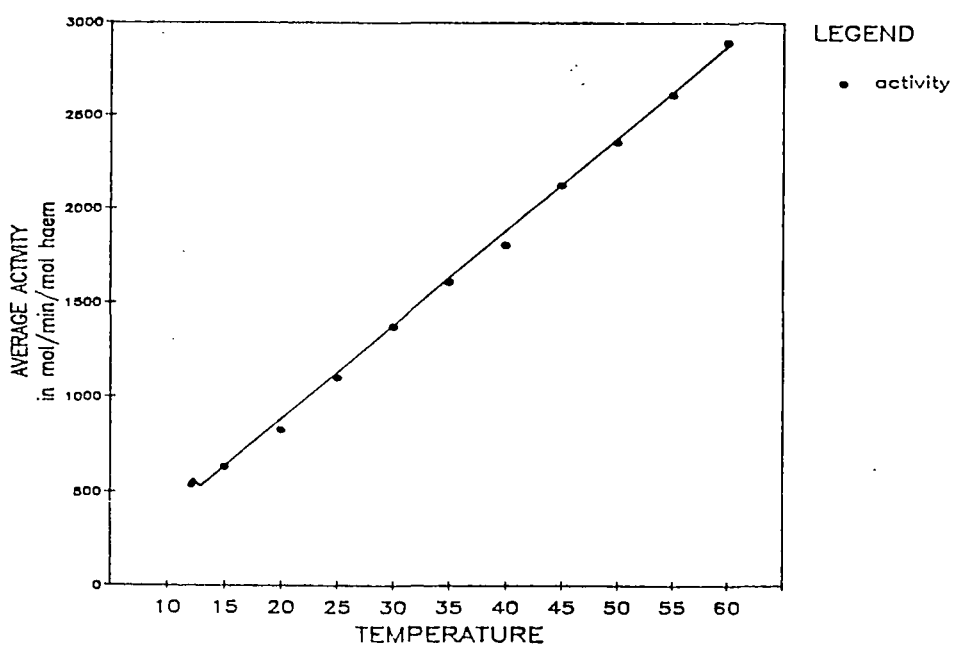


Table 26 The Peroxidase Activity of Pepsin Digested Cytochrome c at Different Temperatures

TEMPERATURE (°C)	AVERAGE ACTIVITY (μmol/min/mg)	(mol/min/mol haems)
10	1.177	20.65
15	2.252	39.53
21	3.090	54.24
25	4.267	74.90
30	3.081	54.08
35	2.716	47.68
40	3.016	52.93
45	2.776	48.73
50	2.728	47.87
54	1.794	31.48
59	1.482	26.01

Fig.31 Temperature Profile of Pepsin Digested Cytochrome c

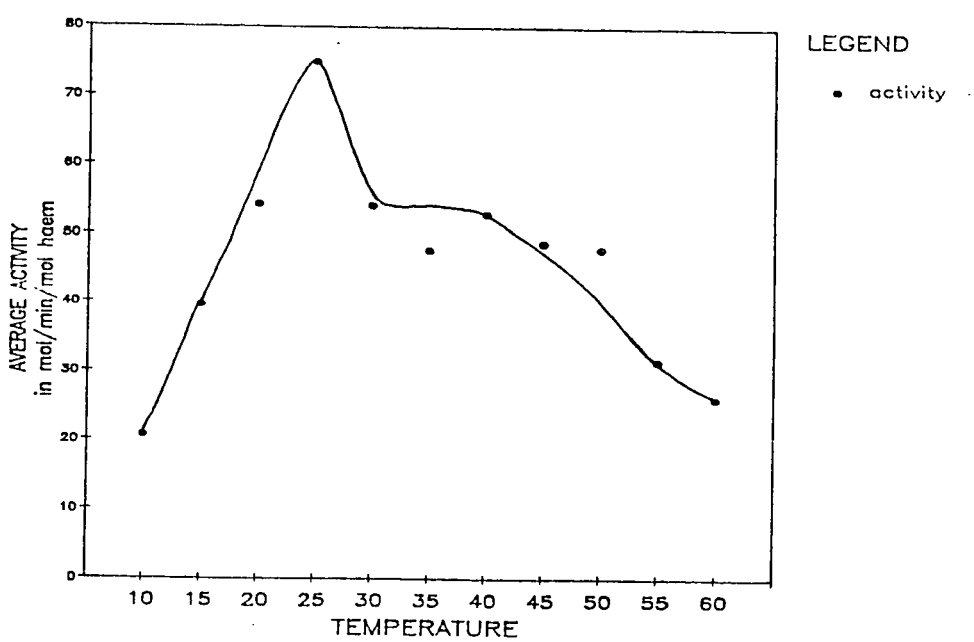
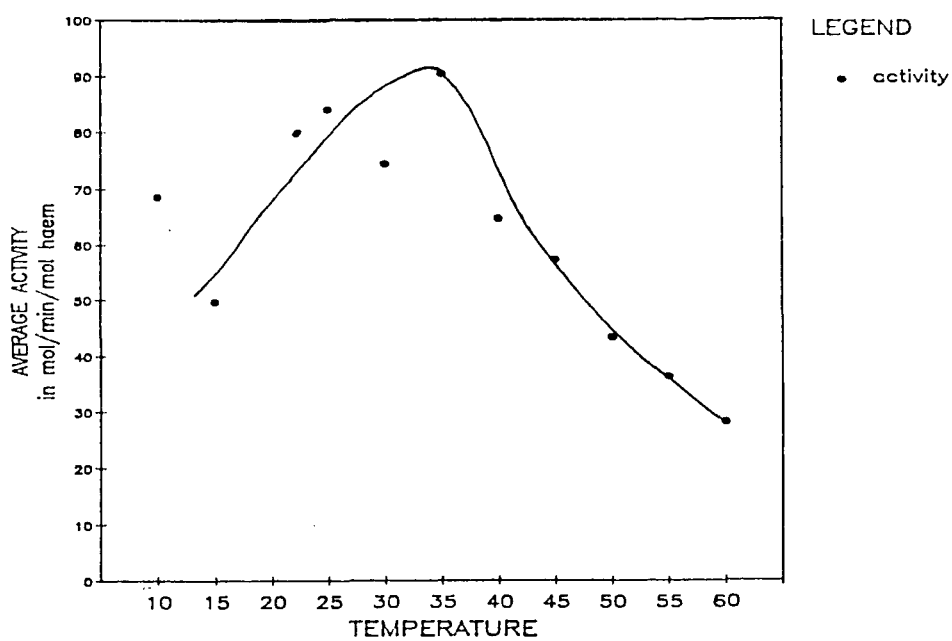


Table 27 The Peroxidase Activity of Trypsin Digested Cytochrome c at Different Temperatures

TEMPERATURE (°C)	AVERAGE ACTIVITY (μmol/min/mg)	(mol/min/mol haems)
10	0.638	68.56
16	0.461	49.52
22	0.741	79.63
25	0.780	83.88
30	0.690	74.21
35	0.840	90.33
40	0.600	64.52
45	0.532	57.21
50	0.402	43.23
55	0.336	36.14
60	0.261	28.02

Fig.32 Temperature Profile of Trypsin Digested Cytochrome c



3.9.0 Peroxidase Activity in Different Media

The guaiacol assay was performed in different media.

Table 28 Peroxidase Activity in Distilled Water

HAEM PROTEIN	ACTIVITY	
	(μmol/min/mg)	(mol/min/mol haem)
mp-11	3.512	25.09
HRP	16.980	1.739×10^{3}
cyt. c	1.83×10^{-3}	0.023
Hb	0.012	0.153
P	1.940	2.638
T	0.742	83.724

(See Table 2 for abbreviations)

The results are expressed in terms of μmol tetraguaiacol formed/min/mg haem protein and mol tetraguaiacol formed/min/mol haem.

Table 29 Peroxidase Activity in Water-Miscible Solvents

SOLVENT	HAEM PROTEIN					
	mp-11	HRP	cyt.c	Hb	P	T
Methanol	++	+++	+		+	+++
Ethanol	++	+++	++			+++
Acetone	++	+++	++			+++
Propan-1-ol	++	+++	+			++
2-methyl propan-2-ol	++	+++		+		
Acetonitrile	++	+++	++		+	
Dimethyl formaldehyde	++		+	+	+	++
Diethylamine	++		+			
Methyl sulphoxide	+++			++	+	
Pyridine	++		+	+		
PEG 300	+			+	+	+

Key

+ = 0.01 - 0.1 mol tetraguaiacol formed/min/mol haem

++ = 0.1 - 1.0 mol tetraguaiacol formed min/mol haem

+++ = 1.0 + mol tetraguaiacol formed/min/mol haem.

(See Table 2 for abbreviations)

3.10.0 Determination of Catalase Activity

Catalase activity was not exhibited by microperoxidase mp-11, horseradish peroxidase, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome c.

3.11.0 Determination of Hydroperoxidase Activity

Table 30 The Hydroperoxidase Activity of Microperoxidase mp-11, Horseradish Peroxidase, Cytochrome c, Haemoglobin, Pepsin Digested and Trypsin Digested Cytochrome c

HAEM PROTEIN	AVERAGE ACTIVITY (µmol/min/mg)	(mol/min/mol haem)
mp-11	0.218	1.557
HRP	ND	ND
cyt.c	1.220×10^{-3}	0.0151
Hb	2.75×10^{-3}	0.0351
P	0.160	2.176
T	0.101	11.396

ND = Not detectable

See Table 2 for abbreviations.

The results are expressed in terms of µmol tetraguaiacol

formed/min/mg haem protein and mol tetraguaiacol formed/min/mol haem.

3.12.0 Halogenation Reactions of Peroxidases

3.12.1 The Oxidation of Iodide

The results are expressed in terms of μmol iodine formed/min/mg haem protein and as mol iodine formed/min/mol haem.

Table 31 Iodoperoxidase Activities

HAEM PROTEIN	AVERAGE ACTIVITY (μmol/min/mg)	(mol/min/mol haem)
mp-11	2.061	14.72
HRP	0.446	45.68
cyt.c	ND	ND
Hb	ND	ND
P	4.574×10^{-3}	0.062
T	ND	ND

ND = Not detectable

(See Table 2 for abbreviations)

3.12.2 The Oxidation of Bromide

The results are expressed in terms of μmol bromine formed/min/mg haem protein and mol bromine formed/min/mol haem.

Table 32 Bromoperoxidase Activities

HAEM PROTEIN	AVERAGE ACTIVITY (µmol/min/mg)	(mol/min/mol haem)
mp-11	0.0698	0.499
HRP	1.652×10^{-3}	0.169
Cyt.c	4.808×10^{-3}	0.060
Hb	2.747×10^{-3}	0.035
P	0.0188	0.256
T	2.735×10^{-3}	0.309

(See Table 2 for abbreviations)

3.12.3 The Halogenation of Monochlorodimedone

The results are expressed as µmol monochlorodimedone utilised/min/mg haem protein and mol monochlorodimedone utilised/min/mol haem.

Table 33 The Chlorination of Monochlorodimedone

HAEM PROTEIN	AVERAGE ACTIVITY (µmol/min/mg)	(mol/min/mol haem)
mp-11	1.965	14.037
HRP	ND	ND
cyt.c	ND	ND
Hb	ND	ND
P	0.157	2.343
T	0.040	4.446

ND = not detected

(See Table 2 for abbreviations)

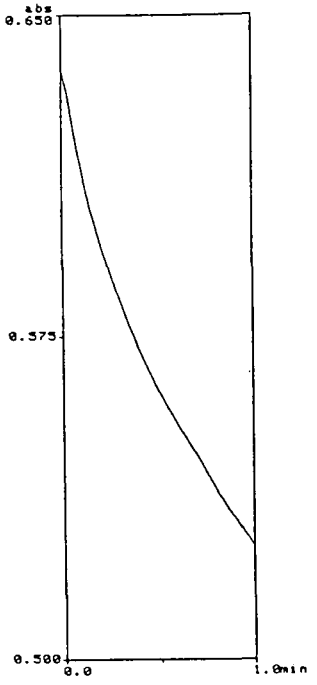
Table 34 The Bromination of Monochlorodimedone

HAEM PROTEIN	AVERAGE ACTIVITY (μmol/min/mg)	(mol/min/mol haem)
mp-11	1.943	13.880
HRP	ND	ND
cyt.c	ND	ND
Hb	ND	ND
P	0.185	2.761
T	0.0427	4.818

ND = Not detected

(See Table 2 for abbreviations)

Fig.33 The Decreases in Absorbance at 278 nm Due to the Disappearance of Monochlorodimedone



3.13.0 The N-Demethylation of Dimethylaniline

A brilliant green solution formed when horseradish peroxidase and N,N-dimethylaniline were mixed in buffer. After the addition of hydrogen peroxide the solution turned yellow-green.

All other reaction mixtures were orange-red.

Table 35 The Absorbance at 421 nm Due to the Production of Formaldehyde

HAEM PROTEIN	ABSORBANCE AT 421 nm
mp-11	0.329
HRP	0.107
cyt.c	-0.160
Hb	-0.065
P	0.142
T	0.018

(See Table 2 for abbreviations)

The blank without a haem protein had an absorbance of 0.009 against water.

Calculation of Demethylase activity

$$\frac{\text{absorbance at 321 nm}}{\text{time}} = \Delta A/\text{min}$$

$$\frac{\Delta A/\text{min}}{\varepsilon} = \text{Molar/min} = C$$

C x reaction volume = moles/min

$$\frac{\text{Moles/min}}{\text{concentration of peroxidase}} = \text{mol/min/mg}$$

ε = molar extinction coefficient. The molar extinction coefficient of formaldehyde = 8000 M^{-1} cm^{-1} at 421 nm (Nash T. 1953)

Reaction volume = 3ml

Table 36 Demethylase Activities

HAEM PROTEIN	AVERAGE ACTIVITY (μmol/min/mg)	(mol/min/mol haem)
mp-11	0.082	0.588
HRP	0.027	2.739
cyt.c	ND	ND
Hb	ND	ND
P	0.036	0.528
T	4.5×10^{-3}	0.507

ND = Not detected

(See Table 2 for abbreviations)

The activity is expressed as μmol formaldehyde formed/min/mg haem protein and mol formaldehyde formed/min/mol haem.

3.14.0 The Hydroxylation of Aromatic Compounds

3.14.1 The Detection of Hydroxylated Aromatics using the Hydroxylated Aromatic Assay and the o-Diphenol Assay

Under the conditions of the experiment, the six haem proteins failed to hydroxylate benzene, benzylalcohol, benzaldehyde, or benzoic acid. The hydroxylated aromatic compound assay was shown to be sensitive enough to detect 2nM α-napthol.

The results using the o-diphenol assay were also negative, however this is only indicative that o-diphenols were not present in the reaction mixture.

A brown precipitate was observed following the addition of freshly hydrated tetrazotised o-dianisidine to the reaction mixture containing horseradish peroxidase and undiluted benzene at pH 6.0.

3.14.2 The Precipitation of ∝-Naphol

A pale brown precipitate was observed when hydrogen peroxide was added to the reaction mixture. This occurred before the addition of the haem protein catalyst and a similar precipitate was not observed when dihydroxyfumaric acid was added to a similar reaction mixture.

For both reactions there was no obvious decrease in absorbance at 530 nm over time when assayed with freshly hydrated tetrazotised o-dianisidine.

The ∝-napthol formed a yellow chromophore in the o-diphenol (Arnow) assay. Both reaction mixtures produced an orange chromophore in the same assay.

3.15.0 The Immobilisation of Horseradish Peroxidase, Pepsin Digested Cytochrome c and Trypsin Digested Cytochrome c

Table 37 An Estimation of the Number of Haems Bound per 10 mg of Each Support

HAEM PROTEIN	No. OF HAEMS/10 mg SUPPORT		
	Glass	Macrosorb	UOP
HRP	1.96×10^{15}	1.18×10^{15}	1.14×10^{15}
P	2.20×10^{16}	6.00×10^{15}	3.07×10^{16}
T	5.12×10^{14}	7.40×10^{14}	2.26×10^{15}

Calculation

(See section 3.5.0 to calculate the number of haems from absorbance values).

Number of haems before immobilisation - number of haems after immobilisation = number of haems bound to 50 mg of support.

Table 38 Approximate Activities of the Immobilised Enzymes

SUPPORT	ACTIVITY (mol/min/mol haem) of IMMOBILISED		
	HRP	P	T
Glass	18.50	1.46	10.61
Macrosorb	31.89	1.09	10.83
UOP	69.71	0.45	2.89

(See Table 2 for abbreviations)

Activity expressed as mol tetraguaiacol formed/min/mol haem.

4.0.0 CONCLUSIONS AND DISCUSSION

4.1.0 The Guaiacol Assay

The results show that horseradish peroxidase has by far the greatest peroxidase activity measured using the guaiacol assay, particularly when the results are expressed in terms of mol tetraguaiacol formed/min/mol haem.

Cytochrome c exhibits very low peroxidase activity with the guaiacol assay, the proteolytic digests of cytochrome c however have considerably higher peroxidase activity (3,000 - 6,000 x greater than cytochrome c). Haemoglobin shows greater peroxidase activity than cytochrome c. (Table 2).

4.1.1 The Effect of Substrate Concentration on the Guaiacol Assay

The results show that a linear relationship exists between the peroxidase activity of microperoxidase mp-11 and the concentration of hydrogen peroxide (between 10-100 mM). (Table 3, Fig. 5).

4.1.2 The Effect of Peroxidase Concentration on the Guaiacol Assay

The peroxidase activity of microperoxidase mp-11 is less in terms of µmol tetraguaiacol formed/min/mg microperoxidase mp-11 when higher concentrations of microperoxidase mp-11 are used in the assay. (Table 4, Fig.6).

This may be explained by the initial reaction rate occurring very rapidly after the hydrogen peroxide was added to the

reaction mixture, but before the spectrophotometer had begun to measure the increase in absorbance at 470 nm.

Peroxidases catalyse the oxidation, by peroxide, of a variety of substances many of which have strong absorption bands or whose oxidation products absorb strongly. Various methods are available for the determination of peroxidase activity:

i) the decrease in hydrogen peroxide;

ii) the decrease in the hydrogen donor;

iii) the formation of the oxidised substrate.

Mostly the third method is applied, the most frequently used donors being guaiacol (o-methyoxyphenol), o-dianisidine and leuco 2, 3'6-trichloroindophenol (Theorell H. 1950, Chance B. and Maehly A.C. 1955, Keilin D. and Hartree E.F. 1955, Herzog V. and Fahimi H.D. 1973, Putter J. 1974, Putter J.and Becker R. 1983).

Two types of specific activity can be measured depending on the experimental conditions.

i) The velocity constant for the formation of the enzyme substrate (ES) complex (eq.16)

$$E + S \xrightarrow{k_1} ES_I \qquad \text{eq.16}$$

ii) The velocity constant for the reaction of the secondary complex with the hydrogen donor molecule (eq.17)

$$ES_{II} + AH \xrightarrow{k_4} E + P \qquad \text{eq.17}$$

Where E = enzyme, S = substrate, P = product..

It is possible to approximate either k, or k4 using guaiacol. The rate of utilisation of peroxide, dx/dt, to form the coloured reaction product measured at 470 nm depends on the

respective concentrations of substrate (peroxide) and donor (guaiacol) (eq.18)

$$dx/dt = \frac{e}{1/k_4 a_o + 1/k_1 x_o} \quad \text{eq.18}$$

where e = enzyme concentration

a_o = initial donor concentration

x_o = initial substrate concentration

k_1 is obtained by choosing the conditions of the assay so that $k_4 a_o >> k_1 x_o$ i.e. the formation of the ES complex is the rate limiting step. If the assay is adjusted so that $k_1 x_o >> k_4 a_o$ then k_4 is obtained. (Chance B. and Maehly A.C. 1955).

Guaiacol is oxidised to tetraguaiacol, a red brown product, but other oxidation products are thought to form under specific conditions. The oxidation of guaiacol by hydrogen peroxide in the absence of peroxidase rarely occurs. The guaiacol solution however is unstable and on ageing there is a photochemical formation of peroxidatic compounds and other reaction products. The peroxidatic compounds act like hydrogen peroxide, while some of the aromatic oxidation products may act as better sustrates than the guaiacol. For this reason only guaiacol solutions less than one week old should be used. (Makinen K. and Tenovuo J. 1982, Putter J. and Becker R. 1983).

Low levels of peroxidase activity can be expressed when employing guaiacol as the hydrogen donor. Very accurate values are obtained with o-dianisidine, but as this compound is potentially carcinogenic its use is limited. A substance originally introduced for the determination of blood glucose, 2,

2'-azino-di-(3-ethylbenzothiazoline-6-sulphonic acid), also gives very accurate results in the determination of peroxidase activity. The peroxidase assay with 3, 3'-diaminobenzidine (DAB) is a simple sensitive and reproducible method for the determination of peroxidase activity. It is claimed to be more sensitive than the guaiacol or o-dianisidine assays, and it is the least carcinogenic of the three compounds. (Herzog V. and Fahimi H.D. 1973, Liu H. and Gibson D.M. 1977, Putter J.and Becker R. 1983).

Different peroxidases react preferably with some hydrogen donors and not with others. The activity should not be compared simply on the results of one hydrogen donor (Theorell H. 1950).

4.2.0 The Proteolytic Digestion of Haemoglobin and Cytochrome c

4.2.1 The Proteolytic Digestion of Haemoglobin

The proteolytic digestion of haemoglobin resulted in an increase in the peroxidase activity of approximately 1.5 x that of the undigested haemoglobin. After four hours of digestion the peroxidase activity was completely lost. The results were similar for each of the four proteolytic enzymes used. (Table 5).

When the pepsin digest was repeated and samples were assayed for peroxidase activity at 5 min intervals, the results show that the peroxidase activity of the pepsin digested haemoglobin was greater at the beginning of the digestion than after one hour of digestion (Table 6, Fig.7).

The increase in peroxidase activity following proteolytic digestion is considerably less for haemoglobin than cytochrome c (section 4.2.2). This may be explained by the way the prosthetic

group is held into the protein. The iron-porphyrin is only loosely associated with the protein in haemoglobin, but is is covalently attached in cytochrome c (Paul K.G. 1955, Margoliash E. and Walasek O.F. 1967, Bendall D.S. et al 1971, Rakshit G. and Spiro T.G. 1974). Thus proteolytic digestion of haemoglobin may result in the haem prosthetic group detaching from the protein. The haemin liberated from the protein is insoluble in neutral aqueous solution and may not have oxidised the guaiacol (Takashi K. et al 1986).

Another possibility for the small increase in activity following digestion may be that the proteolytic enzymes do not expose the haem to such an extent as in the digestion of cytochrome c.

4.2.2 The Proteolytic Digestion of Cytochrome c

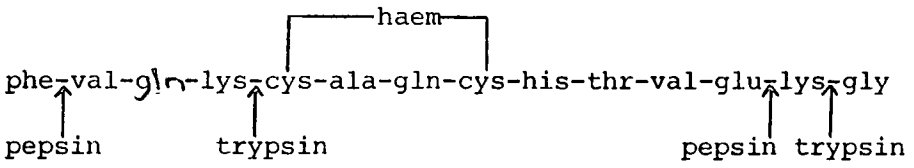
The peroxidase activity of the digests fluctuated with time, only the protease type XXVII digest showed an increase in peroxidase activity with time of digest (Figs.8-11).

The results show that the optimum times for digestion by pepsin, protease type XXVII, trypsin and DPCC -trypsin are 5.5 hr, 6.5 hr, 7.0 hr and 7.5 hr respectively. After 24 hr of digestion the peroxidase activity of the digestion mixtures was showing a decline.

The proteolytic digestion of cytochrome c increases the peroxidase activity by approximately 1,500 - 2,000 fold.

The cytochrome c molecule contains the following potential cleavage sites (Fig.34).

Fig.34 The Potential Cleavage Sites in Cytochrome c



(Plattner H. et al 1977)

Thus digestion of cytochrome c with pepsin produces a fragment containing the haem and an eleven amino-acid peptide, the haem undecapeptide or H-11-P. The digestion with trypsin yields the haem nonapeptide, H-9-P.

Tsou first prepared proteolytic digests of cytochrome c (Tsou C.L. 1951, Margoliash E. et al 1959). Originally the haem oligopeptides were merely by products from enzymatic digestions of cytochrome c for sequence analysis, but their peroxidase activity was recognised by Paleus et al in 1956 (Plattner H. et al 1977).

Alteration of the source of the material or the conditions of hydrolysis permit the isolation of peptides of different composition and slightly different properties. Varying the temperature of digestion and the concentration of the proteolytic enzyme affects the rate of digestion, but ultimately the same product is obtained (Tsou C.L. 1951, Harbury H.A. and Loach P.A. 1960, Peterson J. et al 1983).

Haem undecapeptide has been produced by a number of workers by the peptic digestion of cytochrome c (Tsou C.L. 1951, Margoliash E. et al 1959, Harbury H.A. and Loach P.A. 1960, Tu A.T. et al 1968, Wilcheck M. 1974, Kraehenbuhl J.P. et al 1974, Wilson M.T. et al 1977, Clore G.M. et al 1981, Peterson J. et al 1983). Haem nonapeptide has been produced by passing horse heart

cytochrome c through a column of Sepharose with covalently linked trypsin (Plattner H. et al 1987). The haemoctopeptide, H-8-P is prepared by the trypsin digestion of pepsin digested cytochrome c (eq 19-20).

cytochrome C $\xrightarrow{\text{pepsin}}$ H-11-P Eq.19

H-11-P $\xrightarrow{\text{trypsin}}$ H-8-P Eq.20

(Harbury H.A. and Loach P.A. 1960, Tu A.T. et al 1968, Kraehenbuhl J.P. et al 1974, Peterson J. et al 1983, Baldwin D.A. et al 1985).

Double digests are frequently used in the production of haemoligopeptides (Peterson J. et al 1983).

4.3.0 The Gel Filtration of Pepsin Digested Cytochrome

The separation of a mixture of digested cytochrome c (prepared and lyophyilysed) and undigested cytochrome c were separated on Sephadex G50 using a dissociating buffer (section 3.3.0). However when samples of a digestion mixture were passed through the same column, there was apparently no separation of cytochrome c from digested cytochrome c (section 3.2.1). This would indicate that

i) the cytochrome c has been totally digested to one fragment,

ii) the digested peptides were aggregating to form a larger molecule similar to size to the cytochrome c molecule,

iii) either the digested or undigested cytochrome c was sticking to the column and not eluted.

The latter was shown to be untrue by the PD-10 experiments in which the total activity loaded onto the column and eluted from the column were determined (section 3.3.2).

The disappearance of the absorption peak at 280 nm indicates the absence of tyrosine and tryptophan residues. Cytochrome c gives a typical protein absorption maximum at 280 nm. However following digestion the resulting microperoxidases contain no tyrosine or tryptophan residues, thus the 280 nm peak should be absent. As the 280 nm decreased as the fractions were eluted from the column, this is indicative that some separation of digested and undigested cytochrome c was occurring. (Tu A.T. et al 1968).

Gel filtration is a widely used technique enabling the separation of large and small molecules. Cytochrome c has a molecular weight of approximately 12,000, the haem oligopeptides have a molecular weight of approximately 2,000. Thus separation should be achieved on Sephadex G15 according to their molecular weight.

It has been established that the elution behaviour of proteins is better related to their Stokes radii than to their molecular weights (Le Maire M. et al 1986). Cytochrome c has a Stokes radius of 15 A°, the haem oligopeptides of cytochrome c have a Stokes radius of less than 15 A°, therefore theoretically the digested fragments should be separated from the undigested cytochrome c by gel filtration techniques (Kraehenbuhl J.P. et al 1974).

The haem undecapeptide (H-11-P) has been separated from a pepsin digestion mixture by affinity chromatography using BSA-Sepharose columns (0.5 x 4.0 cm) (Wilcheck M. 1974). Presumably the separation is achieved due to the exposed sixth co-ordination position of the haem oligopeptide binding to the BSA.

The undigested cytochrome c will pass straight through the column as the sixth axial position is co-ordinated to a ligand, thought to be a histidine residue of the protein. (See section 1.1.0).

HPLC with a Biogel P6 (4.0 x 9.0 cm) Column has been shown to separate cytochrome c, H-11-P and H-8-P (Kraehenbuhl J.P. et al 1974). Attempts to separate digested haem oligopeptides from undigested cytochrome c in a digestion mixture by HPLC on a Spherisorb S50D52 (25 x 4.9 cm) column were unsuccessful. The results are not shown in this study, however it was concluded that the haem-containing protein was very hydrophobic.

4.3.1 The Effect of Hydrogen Peroxide and Sodium Dithionite on the Absorption Spectra of Haem Proteins

The results from the gel filtration experiments (3.2.0 and 3.2.1) showed that the absorbance of the fractions in the Soret region shifted gradually towards the blue end of the spectrum with elution time. It was thought to be possible that the haem was being oxidised or reduced as it passed along the column.

Hydrogen peroxide oxidises the haem, forming compound I, two oxidising equivalents above the native haem (section 1.2.1). The addition of hydrogen peroxide to a solution of pepsin digested cytochrome c resulted in a decrease in the absorbance of the Soret peak, but no significant change in position. (Table 12, Fig.16). On the addition of a small amount of sodium dithionite there is a marked increase in the absorbance in the Soret region and a shift in the position of the Soret peak towards the red of the spectrum. (Fig. 17). Sodium dithionite is a reducing agent which reduces the

Fe(III) to Fe(II).

The absorption spectra in the Soret region of reduced and oxidised cytochrome c differ in position and height of the peak (Margoliash E. and Walasek O.F. 1967, Bendall D.S. et al 1971). The Soret band shifts according to derivatives of the iron-porphyrin. The binding of cyanide or azide to the haem proteins is associated with a red shift of the Soret peak and a decrease in its absorbance. (Schoffa G. 1964, Wilson M.T. et al 1977, Blumenthal D.C. and Kassner R.J. 1979).

4.4.0 The Preparation of Microperoxidases

Four digests of cytochrome c were prepared, two each using pepsin and trypsin. Even though the conditions were kept as constant as possible the products from the digestions differed in activity and number of haems/mg (determined in section 2.5.0).

Lyophilisation resulted in a dramatic reduction in activity, in both terms of μmol/min/mg and mol/min/mol haem, even though preliminary experiments (not shown here) indicated that lyophilisation did not reduce the activity of the haem oligopeptides to any significant extent.

The loss of activity following the lyoplilysation of enzymes which are stable in an aqueous environment is often due to the hydrophobic environment. By including sorbitol into the solution prior to lyophilisation, the hydrophobic effects can be overcome (Kennedy J.F. and White C.A. 1985).

4.5.0 The Estimation of the Number of Haem Groups

Cytochrome c, haemoglobin and pepsin digested cytochrome c

posses approximately the same number of haem groups/mg of protein, trypsin digested cytochrome c and horseradish peroxidase possess fewer haems/mg than cytochrome c, microperoxidase mp-11 possess slightly more haems/mg. (Table 14).

As far as the biological activity of the haem protein is concerned, the haem is the active centre of the molecule (Kotani M. 1964). By estimating the number of haems per mg of each protein it enables the peroxidase activity to be calculated in terms of mol tetraguaiacol formed/min/mol haem, thus the peroxidase activity of the haem proteins can be compared.

The calculation is

$$\frac{\text{activity in mol/min/mg}}{\text{no. of haems/mg}} \times 6.022 \times 10^{23} =$$

activity in mol/min/mol haem.

Cytochrome c, horseradish peroxidase and microperoxidase mp-11 have one haem group per molecule. Horseradish peroxidase has the greatest molecular weight (40,000) and therefore it was expected that horseradish peroxidase would have fewer haems/mg than cytochrome c (mw 12,000). The microperoxidases have a molecular weight of approximately 1,600 - 2,000. Therefore 1.0 mg of microperoxidase would be expected to have approximately 6 x the number of haems/mg of cytochrome c. The results however show that there are approximately twice the number of haems/mg microperoxidase mp-11 compared with cytochrome c, and the results also show that the trypsin digested cytochrome c has fewer haems/mg than cytochrome c. This casts doubt on the validity of the method which assumes that the absorbance in the Soret peak is proportional

to the number of haems present. All haem proteins show a characteristic absorbance in the Soret region, the intensity being related to the concentration of the iron-porphyrin present.

The smaller number of haems/mg presence in the microperoxidases may be due to the present of non-haem protein also present in the sample. The number of haems/mg may increase to a value closer to that expected if the microperoxidases could be purified from the digestion mixtures.

4.6.0 The pH Profiles of Microperoxidase mp-11, Horseradish Peroxidase, Pepsin Digested Cytochrome c, and Trypsin Digested Cytochrome c

Microperoxidase mp-11, pepsin digested cytochrome c and trypsin digested cytochrome c have pH optima of 9.0. Horseradish peroxidase has a more neutral pH optimum of 7.5 (Tables 15-21, Figs. 24-28).

Microperoxidase mp-11, pepsin digested cytochrome c and trypsin digested cytochrome c exhibited very low peroxidase activity in acid buffers. The peroxidase activity of microperoxidase mp-11 was affected by the buffer composition, the activity being 9 x lower in glycine buffer than tris buffer at the same pH (Fig.24). With the Zwitterionic Biological Buffers, the activity of microperoxidase was lower in tricine buffer than bis-tris propane buffer at the same pH (Fig. 25). These results suggest that glycine may inhibit the peroxidase activity of microperoxidase mp-11. The different buffer compositions had less effect on the peroxidase activity of horseradish peroxidase.

The effect of the buffer composition on microperoxidases may be due to the ionisation of the amino acids in the peptide chain. Horseradish peroxidase is a large protein, and the ionisation of individual amino acids in the protein will probably be masked. However, microperoxidases have only few amino acids, and charge effects on individual amino acids will be more pronounced.

Although the peroxidase activity of microperoxidase was low in acid, the biocatalysts may be used over a very wide pH range between at least pH 2.6 - 11.8.

Tu et al (1968) Studying the peroxidase activity of haem peptides from horse heart cytochrome c noted that the pH optima for cytochrome c, haem undecapeptide (H-11-P), haem octapeptide (H-8-P) and horseradish peroxidase were different. They found that horseradish peroxidase had the lowest pH optimum of 4.5, cytochrome c, H-11-P and H-8-P had pH optima of 6.0, 9.5 and 7.0 respectively (Tu A.T. et al 1958). Kraehenbuhl et al (1974) also found H-8-P to have a pH optimum of 7.0. Plattner et al (1977) found the pH optimum of haem nonapeptide (H-9-P), produced from the trypsin digestion of cytochrome c, to be 12.5 i.e. a more alkaline pH optimum than the trypsin digested cytochrome c prepared in section 2.4.0. The optimal pH value however depends on the hydrogen donor used (Kraehenbuhl J.P. et al 1974, Plattner H. et al 1977).

4.7.0 The pH Stability of Microperoxidase mp-11 and Horseradish Peroxidase

The results show that after one month microperoxidase

mp-11 and horseradish peroxidase still retain peroxidase activity in buffers pH 4-11 and 6-11 respectively (Tables 22 and 23).

Both microperoxidase mp-11 and horseradish peroxidase were more stable at alkaline pH than acid. Microperoxidase mp-11 retained approximately 97% of its activity at pH 10, horseradish peroxidase retained approximately 86% of its activity at pH 10. There was negligible loss of activity of microperoxidase mp-11 at pH 4, however after one month horseradish peroxidase was no longer active at the same pH.

Cytochrome c seems to be infinitely stable within the pH range 1.7 - 12.3 (Paul K.G. 1955). The haem undecapetide, H-11-P, obtained by pepsin digestion of cytochrome c has been shown to be stable over a wide range of pH values, although in alkaline media it tends to be in varying states of aggregation dependent on pH, temperature and concentration (Margoliash E. et al 1959, Peterson J. and Wilson M.T. 1980, Clore G.M. et al 1981).

Horseradish peroxidase is stable between pH 5.5 - 12.0 in the presence of fluoride, pH 4.5 - 12.0 in the presence of other halogens, azide and cyanide, and pH 3.5 - 12.0 in the absence of the ions listed (Maehly A.C. 1955).

4.8.0 The Temperature Profiles of Microperoxidase mp-11, Horseradish Peroxidase, Pepsin Digested Cytochrome c and Trypsin Digested Cytochrome c

Microperoxidase mp-11, pepsin digested cytochrome c and trypsin digested cytochrome c have temperature optima of 37°C, 25°C and 35°C respectively (Tables 24, 26 and 27, Figs. 29, 31 and 32).

The peroxidase activity of horseradish peroxidase increased linearly with temperature to 60°C (the limit of the water bath used). (Table 25, Fig.30).

These results are the converse of those by Tu et al (1968) who showed that heat treatment of the haem peptides H-11-P and H-8-P does not alter the peroxidase activity, unlike the heat treatment of horseradish peroxidase. They demonstrated that the peroxidase activity of H-11-P and H-8-P increases with temperature following the Arrhenius plot, whereas at higher temperatures the peroxidase activity of horseradish peroxidase decreases due to partial denaturation (Tu A.T. et al 1968).

The thermal stabilities of the haem proteins were not determined. Peroxidases are known to be the most heat stable enzymes in plants. Horseradish peroxidase has been shown to be stable at room temperature for weeks and for 15 min when warmed to 63°C (Maehly A.C. 1955, Muftugil N. 1985).

Although the microperoxidases were shown to have lower temperature optima than horseradish peroxidase, they may be more thermostable. They are certainly suited to carrying out reactions at room temperature, such as the production of fine chemicals which normally occur under harsh conditions and high temperatures.

4.9.0 Peroxidase Activity in Different Media

The peroxidase activity of microperoxidase mp-11, horseradish peroxidase and the two proteolytic digests of cytochrome c (prepared in section 2.4.0) were considerably reduced when the buffer in the guaiacol assay was replaced by distilled

water. The peroxidase activity of haemoglobin was unaffected by the substitution with distilled water, cytochrome c showed greater peroxidase activity when distilled water was used in the assay rather than buffer. (Table 28).

Only microperoxidase mp-11 was capable of peroxidase activity in the eleven water-miscible solvents used in the assay. The peroxidase activity of microperoxidase mp-11, horseradish peroxidase, pepsin digested and trypsin digested cytochrome c were considerably reduced when assayed in solvent not buffer. Only cytochrome c showed a greater peroxidase activity in some solvents than in water. (Table 29).

Microperoxidase mp-11 showed the greatest peroxidase activity in terms of μmol tetraguaiacol formed/min/mg, however in terms of mol tetraguaiacol formed/min/mol haem horseradish peroxidase was the most active peroxidase in solvents.

The experiment was limited to water-miscible solvents due to the problems of assaying the haem proteins in water-immiscible solvents. Kazandjian et al (1986) overcame the problems by evaporating a concentrated solution of peroxidase onto glass powder and using the immobilised peroxidase for assay in water-immiscible solvents. They found that horseradish peroxidase exhibited peroxidase activity in a range of solvents including toluene, benzene, chloroform, ether, n-amylalcohol, although it was necessary to presaturate the ether and amylalcohol with buffer (Kazandjian R.Z. et al 1986).

In many instances it is necessary to determine compounds in organic media, e.g. in industrial chemical reactors and waste

streams, or when analytes are insoluble in water. Enzymes are uniquely suited for analytical applications due to their substrate specificity and are used for the quantitive determination of numerous compounds of clinical, biochemical and industrial interest. So far however, all such analyses have been carried out in aqueous solution. The fact that many enzymes can function in almost anhydrous organic solvents is an important phenomenon, enabling direct enzymatic analysis of compounds in organic solvents (Kazandjian R.Z. et al 1986).

The ability of microperoxidases to function in organic solvents is an important result. Microperoxidases may prove to be useful as biocatalysts for the production of chemical from reagents which are insoluble in aqueous media, by carrying out the reactions in an organic solvent.

4.10.0 Catalase Activity

Catalase activity was not detected in microperoxidase mp-11, horseradish peroxidase, cytochrome c, pepsin digested cytochrome c, or trypsin digested cytochrome c.

The catalase reaction (eq. 21) results in the evolution of oxygen, unlike the peroxidase reaction in which a hydrogen donor is oxidised and oxygen is not released (eq. 22).

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad \text{eq.21}$$

$$H_2O_2 + AH_2 \longrightarrow 2H_2O + A \quad \text{eq.22}$$

(Theorell H. 1950)

Most haematin compounds, including free haematin and iron, possess some oxidative, catalatic and peroxidatic activity (Tsou

C.L. 1951, Keilin D. and Hartree E.F. 1955). The reaction between methaemoglobin and hydrogen peroxide has been shown to bring about the decomposition of hydrogen peroxide to molecular oxygen and water with the accompanying reduction of methaemoglobin to haemoglobin and subsequent oxygenation to oxyhaemoglobin (eq. 23 to 25).

$$\text{metHb} + H_2O_2 \longrightarrow \text{metHb - OOH} \quad \text{eq.23}$$

$$\text{metHb-OOH} \longrightarrow \text{Hb} + O_2 + H_2O \quad \text{eq.24}$$

$$\text{Hb} + O_2 \longrightarrow HbO_2 \quad \text{eq.25}$$

(Keilin D and Hartree E.F. 1950, George P. and Irvine D.H. 1951, Keilin D. and Hartree E.F. 1951).

Proteolytic digestions of cytochrome c have been shown to be devoid of catalase activity (Tsou C.L. 1951, Tu A.T. et al 1968).

4.11.0 Hydroperoxidase Activity

Microperoxidase mp-11, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome showed hydroperoxidase activity. Microperoxidase mp-11 was the most active hydroperoxidase in terms of µmol tetraguaiacol formed/min/mg, but the trypsin digest of cytochrome c (prepared in section 2.4.0) was 7 x more active than microperoxidase mp-11 in terms of mol tetraguaiacol formed/min/mol haem. Horseradish peroxidase did not exhibit hydroperoxidase activity with cumene hydroperoxide as the substrate (Table 30).

Maehly categorises hydroperoxidases as proteins with catalase and peroxidase activity (Maehly A.C. 1952, Walsh C. 1979).

Here the term hydroperoxidase refers to proteins with the ability to reduce organic hydroperoxidases to the corresponding alcohol at the expense of an electron donor (Weller P.E. et al 1985, Maddipati K.R. et al 1987).

Small hydroperoxides such as ethylhydroperoxide and t-butylhydroperoxide are excellent substrates for a broad range of hydroperoxidases. However cumene hydroperoxide, a relatively stable and widely available compound is a poor substrate for horseradish peroxidase, as are fatty acid hydroperoxides. (White R.E. and Coon M.J. 1980, Kedderis G.L. and Hollenberg P.F. 1983, Weller P.E. et al 1985). An excellent substrate for the evaluation of the hydroperoxidase activity of horseradish peroxidase, cytochrome c and lactoperoxidase is 5-phenyl-4-pentenylhydroperoxide (Maddipati K.R. et al 1987).

A broad range of electron donors can be utilised. The most frequently used electron donors are guaiacol, mesidine, aromatic amines and diamines, pyrogallol, iodine, leucodyes, luminol, phenols and reduced cytochrome c (Weller P.E. et al 1985, Maddipati K.R. 1987).

The haem protein undergoes conversion to oxidised intermediates with the reduction of hydroperoxides. The intermediate is thought to be compound I which is two oxidising equivalents above the native ferric form of the enzyme. The formation of compound I occurs with the concomittant release of the alcohol derived from the hydroperoxide. The oxidised intermediate is unable to reduce additional hydroperoxide and a two electron reduction to the native state is required (eq.26 to 28).

P + ROOH ⟶ compound I + ROH eq 26

compound I + AH ⟶ compound II + A eq 27

compound II + AH ⟶ P eq. 28

where P = haem protein, ROOH = organic hydroperoxide, ROH = corresponding alcohol, AH = donor, A = oxidised donor. (Kedderis G.L. and Hollenberg P.F. 1983, Weller P.E. et al 1985).

Hydroperoxide-dependent oxygenations by peroxidases are particularly interesting because they can provide valuable information regarding the mechanism of catalysis (Marnett L.J. and Bienkowski M.J. 1980).

4.12.0 The Halogenation Reactions of Peroxidases

The results show that microperoxidase mp-11, horseradish peroxidase and pepsin digested cytochrome c were able to oxidise iodide to iodine (eq.29) but cytochrome c, haemoglobin and trypsin digested cytochrome c were unable to oxidse iodide to iodine under the conditions of the experiment (Table 31).

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{\text{Peroxidase}} I_2 + 2H_2O \quad \text{eq.29}$$

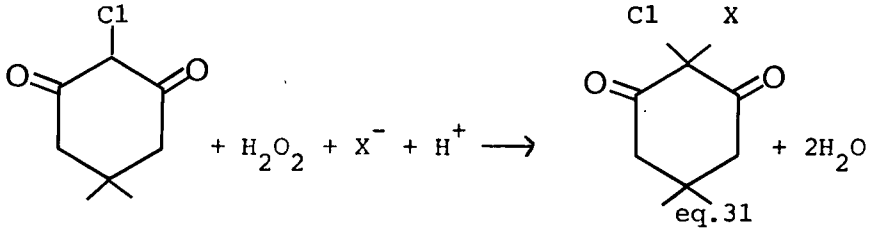
Microperoxidase mp-11 exhibits the greatest iodoperoxidase activity in terms of µmol iodine produced/min/mg but horseradish peroxidase is approximately 3 x more effective at oxidising iodide to iodine than microperoxidase in terms of mol iodine produced/min/mol haem. The pepsin digest of cytochrome c shows very low iodoperoxidase activity.

Unexpectedly all six haem proteins oxidised bromide to bromine (eq.30), although the activities were extremely low. Microperoxidase mp-11 showed the greatest bromoperoxidase activity

in both terms of μmol bromine produced/min/mg and mol bromine produced/min/mol haem (Table 32).

$$2H_2O_2 + 2Br^- + 2H^+ \longrightarrow Br_2 + 2H_2O \quad \text{eq.30}$$

Horseradish peroxidase, cytochrome c and haemoglobin were unable to halogenate monochlorodimedone under the conditions of the experiment. There was very little difference between the rate of chlorination of monochlorodimedone or the bromination of monochlorodimedone by microperoxidase mp-11, pepsin digested cytochrome c and trypsin digested cytochrome c (eq.31). The halogenation of monochlorodimedone by these haem proteins is approximately 10-15 x greater than the oxidation of bromide to bromine by the same haem proteins (Table 33).



eq.31

Peroxidases have long been known to catalyse the oxidation of iodide to iodine using peroxides (Paul K.G. 1955). Horseradish peroxidase is considered to be an iodoperoxidase only and will not oxidise bromide, chloride or the pseudohalide thiocyanate (Morrison M. and Schonbaum G.R. 1976, Libby R.D. et al 1982, Neidleman S.L. and Geigert J. 1985). However the results shown here suggest that horseradish peroxidase has very low bromoperoxidase activity (Table 33).

Horseradish peroxidase failed to halogenate monchlorodimedone in these experiments (Table 34 and 35). However it has previously been shown that although horseradish peroxidase

is unable to oxidise chloride to chlorine, it can chlorinate monchlorodimedone to dichlorodimedone. (Hewson W.D. and Lager L.P. 1979, Frew J.E. and Jones P. 1984).

In the absence of a halogen acceptor such as monochlorodimedone, an intermediate compound, compound X is thought to form. Compound X may be a Fe(IV) form of the enzyme with a chlorine oxide ligand at the haem iron.

It is a matter of contention as to whether the peroxidase catalysed halogenenation involves

i) generation of the halogen or hypohalite followed by non-enzymatic halogenation of the substrate or

ii) formation of an enzyme bound halogenating intermediate (Frew J.E. and Jones P. 1984).

Experimental evidence indicates that the horseradish peroxidase catalysed oxidation of iodide to iodine proceeds primarily via compound I and there is no evidence for the incorporation of iodine in the enzyme (Roman R. and Dunford H.B. 1972).

Morrison and Schonbaum (1976) found three distinct stages in the course of halogenation (eq. 32 to 35).

i) interaction of the peroxidase with the peroxide

$$P + ROOH \longrightarrow \text{compound I} + ROH \qquad \text{eq.32}$$

ii) reduction of compound I to compound II

$$\text{compound I} + I^- \longrightarrow \text{compound II} + I^{\cdot} \qquad \text{eq.33}$$

iii) reduction of compound II to native enzyme

$$\text{compound II} + I^- \longrightarrow P + I^{\cdot} \qquad \text{eq.34}$$

$$I^+ + I^- \longrightarrow I_2 \qquad \text{eq.35}$$

where P = peroxidase, R = H, alkyl or acyl

The overall reaction is

$2I^- + H_2O_2 \longrightarrow I_2 + H_2O$ eq.36

(Roman R. and Dunford H.B. 1972, Morrison M. and Schonbaum G.R. 1976).

Peroxidase catalysed halogenations can be used very conveniently to obtain a label of high specific activity in proteins. The iodination of proteins has been a widely used tool for many years to

i) study the molecular structure of protein

ii) label proteins to investigate their metabolism

iii) increase the sensitivity of assay procedures in radioimmune assays.

Peroxidase catalysed iodination is advantageous is that it has fewer side reactions than halogenation employing other reagents and under the correct conditions, peroxidase catalysed halogenations can be very selective, easily controlled and readily followed during the course of the reaction (Morrison M. and Schonbaum G.R. 1976).

The microperoxidase biocatalyst could be used to produce novel halogenated compounds.

4.13.0 N-Demethylation Reaction of Peroxidases

The results show that microperoxidase mp-11, horseradish peroxidase, pepsin digested cytochrome c and trypsin digested cytochrome c (Section2.4.0) possess demethylase activity (Table 36).

The demethylase activities are very low compared to the

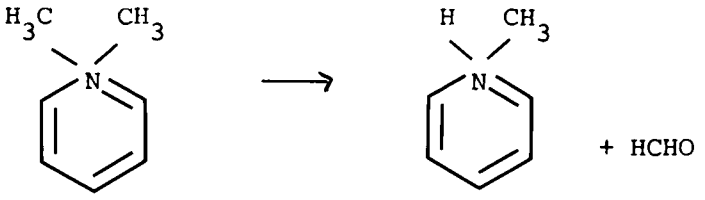
peroxidase activities of the same haem proteins. The demethylase activity of microperoxidase mp-11 is 100 x less than its peroxidase activity, horseradish peroxidase has a demethylase activity more than 500 x lower than its peroxidase activity. Haemoglobin and cytochrome c were unable to demethylate dimethylaniline under the conditions of this experiment.

The hydrogen-peroxide dependent N-demethylation of several drug substrates of cytochrome P_{450} by horseradish peroxidase was first reported in 1958 by Gillette et al (1958). More recently the hydrogen peroxide dependent N-dealkylation of a number of N, N-dimethyl and N, N-dibutylaniline by horseradish peroxidase have been demonstrated (Galliani G. et al 1978, Kedderis G.L. and Hollenberg P.F. 1983).

The N-demethylation of aminopyrine has been shown to be catalysed by horseradish peroxidase and methaemoglobin in the presence of hydrogen peroxide. Horseradish peroxidase is 1000 x more active than methaemoglobin (Griffin B.W. and Ting P.L. 1978).

The N-demethylation of dimethylaniline has been catalysed by horseradish peroxidase, microperoxidase, haemoglobin, methaemoglobin and cytochrome c. The ethylhydroperoxide or hydrogen peroxide supported N-demethylation of N, N, -dimethylaniline by horseradish peroxidase results in the formation of equimolar amounts of N-methylaniline and formaldehyde with no other products detectable by HPLC (eq.37).

N,N-dimethylaniline $\xrightarrow{\text{peroxidase}}$ N-methylaniline + formaldehyde eq.37



Kinetic evidence of the reaction is consistent with a ping-pong mechanism (Kedderis G.L. et al 1980, Kedderis G.L. and Hollenberg P.F. 1983). In the demethylation of aminopyrine, it has been shown that the aminopyrine can function as an electron donor to both compound I and compound II of horseradish peroxidase (Griffin B.W. and Ting P.L. 1978).

Horseradish peroxidase and hydrogen peroxide are able to bring about the O-demethylation of the cytotoxic agents 9-methyoxyellipticine and N,N-methyl-9-methoxy-ellipticinium acetate. The reaction results in the formation of the corresponding quinone-imine derivatives with the concommittant formation of one molecule of methanol per molecule of methoxy compound.

The peroxide catalysed apparent O-demthylation has been demonstrated to be an oxidative demthylation. In oxidation reactions the enzyme is concerned with abstracting electrons from easily oxidisable compounds. The oxygenation reactions, for example of cytochrome P_{450}, involve the incorporation of oxygen into the substrate (Meunier G.and Meunier B. 1985).

The greater demethylase activity of peroxidases suggests these reactions may be useful as model systems for microsomal

demethylation reactions of drugs (Kedderis G.L. and Hollenberg P.F. 1983).

4.14.0 The Hydroxylation of Aromatic Compounds by Peroxidases

The attempts to hydroxylate various aromatic compounds using microperoxidase mp-11, horseradish peroxidases, cytochrome c, haemoglobin, pepsin digested cytochrome c and trypsin digested cytochrome c were unsuccessful. This may be due to the inability of the haem peroxidases to hydroxylate aromatic compounds under the conditions of the reactions or the assays used to detect the hydroxylated aromatic compounds were unsuitable or insufficiently sensitive.

The hydroxylated aromatic assay (Wackett L.P. and Gibson D.T. 1983) failed to produce a violet colouration with phenol, and therefore it may have failed to detect hydroxylated aromatic a reaction products if they were present.

The o-diphenol (Arnow) assay produces a bright red chromophore with simple and monosubstituted o-diphenols. Monomethyl catechol ethers and 2,3-dihydroxypyridine react to form yellow-orange products, however di- and tetra- substituted o-diphenols, monophenols, m- and p- diphenols do not produce visible reaction products in this assay (Waite J.H. and Tanzer M.L. 1981). It is possible therefore that hydroxylated aromatic compounds were being produced in the reaction mixtures but they could not be detected by this assay.

The brown precipitate observed when tetrazotised o-dianisidine was added to the reaction mixture and left to stand

at room temperature for a few minutes may be the result of a reaction between benzene with the tetrazotised o-dianisidine, or it may be a polymerisation product of benzene. The latter would suggest that the benzene had been hydroxylated and the products of hydroxylation had polymerised.

The reaction of α-napthol with the o-diphenol to produce a yellow reaction product was unexpected. The reaction mixtures produced an orange product in the same assay and this may be due to the hydroxylation of α-napthol.

The hydroxylation of aromatic compounds using horseradish peroxidase has been previously demonstrated (Halliwell B. and Ahluwalia S. 1976, Halliwell B. 1977, Dordick J.S. et al 1986). The reaction products in these experiments were detected by HPLC analysis of the reaction mixtures.

Mason and co-workers (1957) reported the hydroxylation of a number of aromatic compounds catalysed by horseradish peroxidase in the presence of reducing equivalents and oxygen. No peroxidase is known to perform aliphatic hydroxylations (White R.E. and Coon M.J. 1980, Dordick J.S. et al 1986).

The hydroxylation of aromatic compounds will not occur during the usual peroxidase reaction. The hydroxylations will only take place in the present of dihydroxyfumaric acid and oxygen. The oxygen is indispensable as hydroxylation will not occur under nitrogen. The dihydroxyfumaric acid in the reaction mixture cannot be replaced by equimolar concentrations of other reducing equivalents such as ascorbate, cysteine, sulphite, NADH or hydrogen peroxide. The addition of hydrogen peroxide to

oxygen-dihydroxyfumaric acid/peroxidase mixtures accelerates the rate of hydroxylation without affecting the final yield (Paul K.G. 1963, Halliwell B and Ahluwalia S. 1976, Halliwell B. 1977, Dordick J.S. et al 1986).

When oxidising dihydroxyfumaric acid (DHFA), the peroxidase becomes converted to an oxygenated ferriperoxidase, known as oxyperoxidase or compound III. Compound III decays to yield the superoxide anion, hydrogen peroxide and the ferrienzyme with the concomittant hydroxylation of aromatic compounds in the reaction mixture. It has been proposed that the superoxide radicle and hydrogen peroxide formed during the oxidation of dihydroxyfumaric acid combine together forming the hydroxyl radicle (eq.38 to 41).

$$DHFA + O_2 \longrightarrow [x]^{\cdot} + O_2^{-\cdot} \qquad \text{eq.38}$$

$$2H^{+} + O_2^{-\cdot} + DHFA \longrightarrow [x]^{\cdot} + H_2O_2 \qquad \text{eq.39}$$

$$[x]^{\cdot} + O_2 \longrightarrow \text{dioxosuccinate} + O_2^{-\cdot} \qquad \text{eq.40}$$

$$O_2^{-\cdot} + H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} + O_2 \qquad \text{eq.41}$$

where $[x]^{\cdot}$ is the free radicle formed by the loss of the electron from DHFA.

The hydroxyl radicle is required by the system for the hydroxylation reaction since scavengers of the hydroxyl radicle inhibit the hydroxylation of aromatic compounds (Halliwell B. and Ahluwalia S. 1976, Halliwell B. 1977, Dordick J.S. et al 1986). Hydrogen peroxide is thought to be important in haemoglobin catalysed hydroxylations (Dordick J.S. et al 1986).

The following aromatic compounds have been hydroxylated

phenol $\longrightarrow$ catechol

catechol ⟶ o-quinone

salicylate ⟶ 2, 3- and 2, 5- dihydroxybenzoate

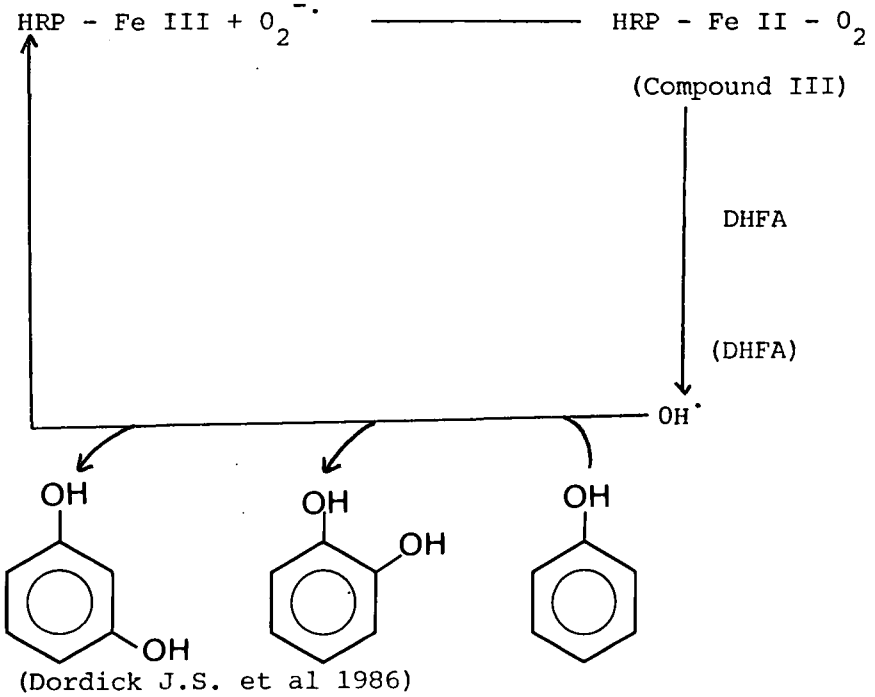
cinnamate ⟶ 2- and 4- hydroxyacids

p-coumarate ⟶ caffeate

(Paul K.G. 1963, Halliwell B. and Ahluwalia S. 1976, Halliwell B. 1977, Dordick J.S. et al 1986).

Dordick et al (1986) propose the following scheme for aromatic hydroxylations by horseradish peroxidase (Fig. 35).

Fig. 35 Scheme for the Hydroxylation of Aromatic Compounds



(Dordick J.S. et al 1986)

The horseradish peroxidase catalysed hydroxylation of aromatic compounds is an activity quite different from the typical reaction catalysed by the enzyme in the presence of aromatic compounds, namely the peroxidative coupling of aromatic amines and phenols. The major distinction involves the inability of hydrogen

peroxide to support hydroxylations (Dordick J.S. et al 1986).

4.15.0 The Immobilisation of Peroxidases

The results show that horseradish peroxidase, pepsin digested cytochrome c and trypsin digested cytochrome c were immobilised in an active form on controlled pore glass beads, Macrosorb K (GA) and UOP immobilisation supports. (Table 38).

The activity of the immobilised haem proteins however is markedly lower than the free forms. The activity of horseradish peroxidase immobilised on glass is approximately 100 x lower than the free enzyme. Interestingly the peroxidase activity of horseradish peroxidase was greater on UOP > macrosorb > glass whereas the converse was true for the small haem oligopeptides from cytochrome c.

Over the last few years the number of methods available for enzyme immobilisation have increased dramatically. There are various ways of classifying the several types of immobilised enzymes. The term immobilised enzyme includes

i) enzymes modified to a water insoluble form by suitable techniques;

ii) soluble enzymes used in reactors equipped with semi-permeable ultrafiltration membranes, allowing the passage of reaction products resulting from the hydrolysis of high molecular weight substances but retaining the enzyme molecules inside the reactor;

iii) enzymes, the mobility of which has been restricted by their attachment to another macromolecule, but the overall composite molecule remains water soluble. (Kennedy J.F. and White C.A. 1985).

The attachment of an enzyme to an insoluble carrier is the oldest and most prevalent method for enzyme immobilisation. Physical absorption is an attractive method of immobilisation owing to the mild conditions involved, however the binding forces are generally weaker than chemical binding methods and operational stabilities are lower. Chemical reactions, such as cross-linking and covalent binding and conformational changes, must be kept to a minimum. (Kennedy J.F. and White C.A. 1985).

With the small haem peptides, cross-linking of the peptides would probably result in the haem group being obscured, defeating the object of exposing it by proteolytic digestion. However, attaching the peptide to a support leaving the haem free and exposed has the possibility of reducing aggregation and improving the activity of the microperoxidases.

The specific activity of an enzyme frequently decreases on immobilisation. The diffusion of the substrate from the bulk solution to the microenvironment of an immobilised enzyme may be a major factor in the rate of enzyme reaction. Shifts in pH optima have been noted on the immobilisation of many enzymes. (Kennedy J.F. and White C.A. 1985).

Inorganic carriers may generally be expected to possess superior properties over their organic counterparts in relation to mechanical strength and biological stability, the possibility of reuse, surface properties and flow and mass transfer characteristics (Cabral J.M.S. et al 1981).

Although the utilisation of immobilised enzymes in clinical chemistry and environmental analysis is in rapid progress,

much of the application and use of immobilised enzymes has been on a small scale (Mattiasson B. et al 1978, Kennedy J.F. et al 1981).

Immobilised enzymes are very important for biocatalysis in reactors. The immobilisation of microperoxidase is an important result regarding the possible use of the biocatalyst.

5.0.0 SUMMARY

This study has examined some of the properties of microperoxidases. The results show that

i) microperoxidases are extremely easy to prepare, although there are problems purifying them by gel filtration,

ii) they are active, and stable, over a wide range of pH with a very alkaline pH optimum,

iii) they are active up to at least 60°C, with a temperature optimum of 25-37°C. Thus reactions can be readily carried out by microperoxidases at room temperature,

iv) they are capable of exhibiting peroxidase activity in a wider range of solvents than horseradish peroxidase (which is one of the most active enzymes known),

v) they can perform other hydrogen peroxide - dependent reactions such as demethylations and halogenations, with greater ease in some instances than horseradish peroxidase.

Microperoxidases have been used academically as model systems to investigate the haem group, ligand binding and the role of protein in catalysis. They have been used as tracers in electron microscopy, and in immunoassays. Their potential as biocatalysts for the production of fine chemicals has yet to be fulfilled.

Further research is required to

i) optimise the production of haem oligopeptides with the greatest peroxidase activity,

ii) to investigate the possible production of microperoxidases from other cytochromes, particularly cytochromes of bacterial

origin,

iii) to purify the microperoxidases from undigested cytochrome c in the digestion mixture, maybe using HPLC or an affinity method,

iv) to determine the temperature stability of microperoxidases,

v) to investigate the range of chemistry which microperoxidases are capable of.

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