STUDIES ON NATIVE AND MODIFIED PEROXIDASES.

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I hereby declare that the research described within this thesis is based entirely upon my own work.

Orlaith Ryan, B.Sc.,
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ABSTRACT

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The aim of the project was to chemically modify horseradish peroxidase (HRP) in an attempt to enhance the stability of the enzyme. Initially a colorimetric microassay for horseradish peroxidase was developed. Chemical modification of the enzyme was then carried out using various homo- and hetero- bifunctional crosslinking reagents in an attempt to improve the stability and hence the shelf life and range of applications of horseradish peroxidase. Stabilized derivatives were produced using bis-imidates and N-hydroxy bis-succinimide esters. Bis-imidates are amino specific homobifunctional crosslinking reagents. Successful crosslinking was seen using dimethyl suberimidate (molecular length = 1.1nm), dimethyl adipimidate (0.77nm) and to a lesser extent for dimethyl pimelimidate (0.92nm). Increased thermostability was used as an index that crosslinking had occurred. Crosslinking prevents unfolding of the protein backbone and so a crosslinked enzyme should not unfold when heated. Thermostability was assessed by incubating native or modified enzyme at 72.5°C for 60 minutes. Enzyme activity of samples withdrawn onto ice over the 60 minute incubation period was determined using standard conditions to see if thermostability had been conferred by crosslinking. Although increased thermostability was seen for bis-imidate treated samples, after 60 minutes at 72.5°C modified samples were only slightly more active than native enzyme. N-hydroxy-succinimide esters were also used to modify HRP. Very successful results were seen with these homobifunctional reagents. Increased thermostability was seen to the level that modified samples only lost a small degree of activity after 60 minutes at 72.5°C. Characterization of modified HRP was carried out to determine the effects of modification on the properties and structure of the enzyme. In all cases there was no adverse effect on catalytic activity and no physical alterations to the enzyme were evident. Thus, chemically-stabilized derivatives of HRP have been produced.

ABBREVIATIONS

$\overset{\circ}{A}$	Angstrom
ABTS	2,2'-azino-bis-[3-ethyl benzthiazoline]-6-sulphonic acid
Ag/AgCl	Silver/Silver chloride
AH_2	Hydrogen acceptors
CD	Circular Dichroism
CO_2	Carbon dioxide
4-CN	4-chloro-1-naphtol
CHEMFET	FET containing a chemically sensitive layer
CN^-	cyanide
DAB	3,3'-diaminobenzidine
DH	Hydrogen donor
DMA	Dimethyl adipimidate
DMP	Dimethyl pimelimidate
DMS	Dimethyl suberimidate
DMSO	Dimethyl sulfoxide
DSC	Differential Scanning Calorimetry
DTNB	5,5' dithio-bis-[2-nitrobenzoic acid]
EDA	Ethylene diamine
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EG-NHS	Ethylene glycol bis-succinic acid ester of N-hydroxysuccinimide
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
ESR	Electron spin resonance
FET	Field Effect Transistor
FT-IR	Fourier Transform-Infra Red
ΔG	Gibbs energy
GMBS	γ-maleimididobutyric acid N-hydroxysuccinimide ester
GnCl	Guanidine hydrochloride
H_2O_2	Hydrogen peroxide

HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
HS-(CH$_2$)$_n$-SH	Dithiol
H$_2$SO$_4$	Sulphuric acid
I$^-$	Iodide
ISE	Ion-selective electrode
ISFET	Ion-selective field effect transistor
2-IT	2-Iminothiolane
LED	Light emitting diode
MAI	Methylacetimidate
MBTH	3-methyl-2-benzothiazoline hydrazone hydrochloride
MES	2-[N-morpholino]ethane sulphonic acid
MOSFET	metal oxide semi-conductor field effect transistor
N	Native, folded protein
Na$_2$B$_4$O$_7$	Sodium tetraborate
NaH$_2$PO$_4$	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
`NH$_2$	Amino group
NH$_3$	Ammonia
NH$_4^+$	Ammonium
NHS	N-hydroxy succinimide
4NM	4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxy succinimide ester
NMP TCNQ	N-methylphenazinium tetracyanoquinonodimethanide
NMR	Nuclear Magnetic Resonance
O$_2$	Oxygen
OPD	o-phenylene diamine
ORD	Optical Rotatory Dispersion
PEG	Poly ethyleneglycol
POPHA	p-hydroxy-phenylacetic acid
SA-NHS	Suberic acid bis-ester of N-hydroxy succinimide
SDS	Sodium dodecyl sulphate

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SO_2	Sulphur dioxide
SPDP	N-succinimidyl 3-(2-pyridyldithio) propionate
TCNQ	Tetracyanoquinodimethane
TEMED	N,N,N',N'-tetramethylethylenediamine
TMB	3,3',5,5'-tetramethylbenzidine
TNB	thio(2-nitrobenzoic acid)
TNBS	Trinitrobenzenesulphonate
Tris HCl	Tris[hydroxymethyl]aminomethane hydrochloride
TTF	Tetrathiafulvalene
TTF TCNQ	Tetrathiafulvalinium tetracyanoquinodimethanide
U	Denatured, unfolded protein
U.V.	Ultraviolet
X	Inactivated protein

1. HORSERADISH PEROXIDASE: FUNCTION AND APPLICATIONS

1.1. INTRODUCTION:

The biochemistry of horseradish peroxidase will be reviewed in this chapter. Details on reaction schemes, structural components and on various properties and recent studies will be included. Horseradish peroxidase (HRP) is frequently used as a model enzyme in chemical modification studies. The rationale behind chemical modification is discussed and properties of stabilized enzymes and their specific applications, in particular of modified HRP, are considered. A specific area where HRP is used frequently is in biosensors. A general review on biosensors is presented, including examples in which HRP is employed.

1.2. BIOCHEMISTRY OF HORSERADISH PEROXIDASE:

1.2.1. History and Distribution:

Peroxidases were first observed in 1855 by Schoenbein. For the remainder of the 19th century many novel preparations, theories and reaction conditions relating to peroxidases, or purported peroxide utilizing preparations were put forward, as outlined by Paul (1963). In the 1930's peroxidase preparations of quite high purity were obtained from fig sap and from horseradish. Horseradish (*Armoracia rusticana*), is one of the richest sources of peroxidase, and its peroxidase was crystallized by Theorell in 1942 (See Paul, 1963). Horseradish peroxidase (HRP) was the fourth haemoprotein to be crystallized, succeeding haemoglobin, myoglobin and catalase. The next few decades brought details about specific peroxidase reactions and novel sources. Horseradish peroxidase, as the name implies, is a plant peroxidase. Peroxidases also occur in human saliva, adrenal medulla and in the liver, kidney and leucocytes. The importance of peroxidase in living cells is in participation in coupled oxidations and in safeguarding the cell against peroxide poisoning. Peroxidase reactions in microorganisms are rare. Yeast has been shown

to contain cytochrome c peroxidase. All plant peroxidases purified up until Paul wrote his review in 1963 possess protohaem as prosthetic group. Plant peroxidases appear to be less specific than other peroxidases with regard to the type of compound they oxidize. Some peroxidases, for example, from yeast and liver, act specifically on one type of oxidizable compound. Peroxidases catalyze the oxidation of certain compounds (AH_2) by peroxide. The protohaem peroxidases react with uncharged AH_2 species. Flavoprotein peroxidases differ from the protohaem peroxidases with respect to inhibitors and reaction with AH_2. Both types of peroxidases generate a product in which a hydrogen atom is removed or an electron is removed after the previous loss of a proton (Paul 1963). In 1976, a peroxidase was reported from a previously unknown source, i.e., from human cervical mucus (Shindler et al, 1976). The properties and kinetics of this peroxidase are similar to a classical peroxidase and the spectral properties of human cervical mucus peroxidase indicate that it is a haemoprotein. The mechanism of action of this peroxidase was investigated in full and revealed a complex kinetic scheme of reaction, similar to other peroxidases. In human secretions, peroxidases occur in saliva, tears, milk and cervical mucus and are found in certain body tissues. Human salivary peroxidase products are involved in the regulation of growth and metabolism of oral bacteria (Pruitt et al, 1990).

1.2.2.   Isoenzymes:

Peroxidase isoenzymes are present throughout the plant kingdom. The C isoenzyme of HRP dominates to a large extent. Several observations of multiple components of HRP with identical spectra or activities have been reported. Peroxidase isoenzymes were first detected by Theorell in 1942 in horseradish roots. These isoenzymes, separable by salt precipitations, paper electrophoresis, or chromatography were isolated and characterized into two groups by Shannon et al (1966). They isolated seven peroxidase isoenzymes from horseradish roots by the purification procedures of ammonium sulphate precipitation and column chromatography on carboxymethyl cellulose and DEAE-cellulose. Purification procedures yielded a high recovery of enzyme activity. Peroxidase activity in the

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seven isoenzymes accounted for 86% of the original enzyme activity. The purity of the seven isoenzymes was ascertained by chromatography, ultracentrifugation and polyacrylamide disc gel electrophoresis. After several chromatographic separations, the elution profile of each isoenzyme yielded a single protein peak which co-eluted with peroxidase activity. These elution profiles were reproduced a number of times. This and the fact that no interconversion of isoenzymes was detected during the purification procedure indicate that the components isolated were genuine isoenzymes. Ultracentrifugation of the isoenzymes yielded sedimentation coefficients that were very similar. The testing of seven isoenzymes for homogeneity by polyacrylamide disc gel electrophoresis revealed that each isoenzyme migrated as a single protein band. Purity of the seven isoenzymes isolated by Shannon et al was confirmed by the results of the chromatographic studies and by the results from ultracentrifugation and electrophoresis, described above. Spectrophotometric studies of the isoenzymes showed two distinctive absorption spectrum patterns, one for each group of isoenzymes. Use of different pH values and different buffers had a profound influence on the absorption spectra. For some of the isoenzymes, there was a reversible shift in the peak of the Soret band from 401 nm, in response to pH. At a given pH the peak of the Soret band varied in both intensity and position, depending on the type of buffer in which the enzyme was dissolved. These findings in relation to changes in absorbance spectra may explain the reports in the literature of two types of HRP. Plant peroxidases possess protohaemin IX as the prosthetic group and the isolated peroxidases are no exception. The haemin isolated from each isoenzyme cochromatographed with genuine protohaemin IX.

Carbohydrate is present in each of the seven isoenzymes. 18% of the HRP is accounted for by carbohydrate, i.e., HRP is a glycoprotein. Each isoenzyme isolated by Shannon et al contains carbohydrate as an integral part of the molecule. Since peroxidase activity has been demonstrated in the absence of the carbohydrate moiety, it seems unlikely that carbohydrate participates directly in catalysis. The seven isoenzymes may be segregated into two groups on the basis of some specific properties. The charge characteristics and spectrophotometric behavior of the seven isoenzymes fall into two main groups. A similar grouping is seen in the differences

in carbohydrate and amino group composition. The basic nature of one of the groups of isoenzymes can be attributed to a high content of the basic amino acid, arginine. The other group contains neutral and acidic residues. Electrophoretic mobility falls into two groups as do all the other properties studied. Morita et al (1982) crystallized the basic isoenzyme of HRP. Although HRP itself and its various isoenzymes have been crystallized, the difficulty in making suitable crystals of HRP for X-ray cystallography means that the three-dimensional structure of the enzyme has not yet been determined. The extreme difficulty in making suitable crystals of plant peroxidases seems to be due to the carbohydrate content of these enzymes (Morita et al, 1982).

1.2.3. Main Features of Horseradish Peroxidase Molecule:

Horseradish peroxidase is an oxidoreductase (Donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7). It is a haemoprotein that transfers hydrogen from hydrogen donors (DH) to H_2O_2, as do all peroxidases (Tijssen, 1985). Horseradish peroxidase isoenzyme C is a single polypeptide chain consisting of a haemin prosthetic group and 308 amino acid residues, including 4 disulphide bridges and 2 Ca^{2+} ions. These properties and the amino acid sequence of horseradish peroxidase isoenzyme C were elucidated by Welinder (1979). In addition this single chain enzyme contains 8 neutral carbohydrate side chains. The details given above for isoenzyme C are true for all other isoenzymes of horseradish peroxidase. The molecular weight of native HRP, a glycoprotein, is 44,000. One mole of protohaemin IX per HRP molecule acts as a prosthetic group. This protoporphyrin IX group is held in place by electrostatic interactions between the propionic side chain of the haem and a lysine molecule in the apoprotein. The covalent structure of HRP isoenzyme C consists of two compact domains, between which the haemin group is positioned (Welinder, 1979). The iron group has six co-ordinate positions, of which four are occupied by porphyrin nitrogen atoms and the fifth by a protein group. The sixth can be occupied by various compounds. It is by exchange of substrate in this 6th position that peroxidases appear to operate (Tijssen, 1985). (See Fig. 1.1).

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Peroxidase

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                    ┌──────────────────────┐
        protohaematin IX (active site)      glycoprotein
        ┌──────────────┐                         │
      Iron      protoporphyrin IX          (colourless
                                            apoprotein)
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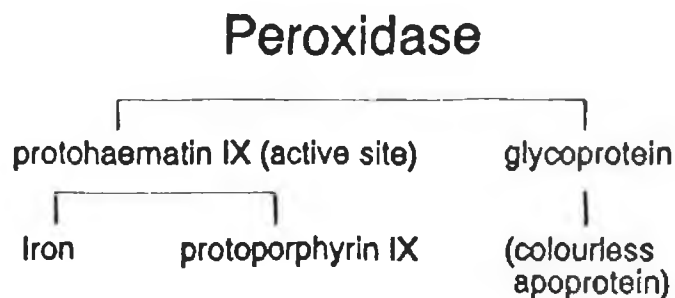
Fig. 1.1.   Components of the peroxidase molecule.

The carbohydrate side chains are attached at asparagine residues (Welinder, 1979) at 8 different sites. Carbohydrates are more abundant in the C terminal region of the enzyme molecule. The characteristic absorption spectrum of native peroxidase shows a major Soret band at 403nm. Horseradish peroxidase contains a single tryptophan residue, that fluoresces, which is not located in the same domain as the active site. Horseradish peroxidase activity is measured indirectly by the rate of transformation of the hydrogen donor. The pH optimum of HRP is 4.0 - 8.0 (Paul, 1963). Specific formation of compound I, as shown in Fig 1.2, can only be obtained with certain peroxides. The specific hydrogen acceptors that can be used are hydrogen-, methyl- and ethyl peroxide. Only these three hydrogen acceptors are active, whereas a wide range of hydrogen donors react (Tijssen, 1985). These include a large number of phenols, aminophenols, indophenols, diamines and leuco dyes. The reaction of various hydrogen donors and other substrates with horseradish peroxidase will be discussed later in Section 1.2.8.

1.2.4.   Catalysis:

The reaction scheme for peroxidase catalysis is shown in Fig. 1.2. Peroxidase combines with and is oxidized by hydrogen peroxide (substrate) to form component I. It is the haem group of the peroxidase that is oxidized. In the absence of suitable

oxidized donor ←⊤→ peroxidase

⌡⌠ H_2O_2 or other substrates

compound I

one electron transfer ↓   hydrogen donor (DH)
                                 ↓
                         oxidized donor (D)

hydrogen donor ←⌐→ compound II

excess of substrate

↓

compound III and IV

(inactive)
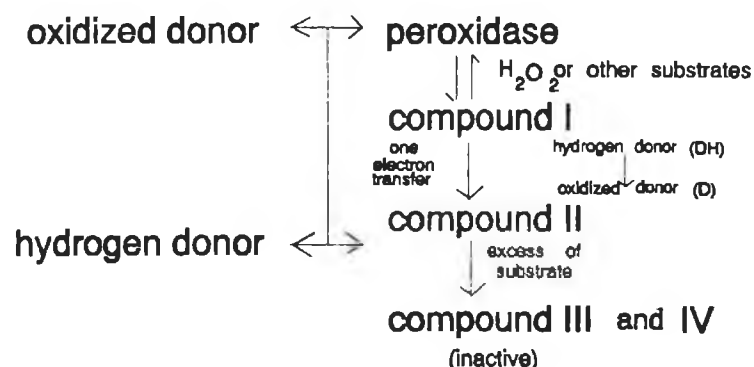
Fig. 1.2.   Scheme of catalysis of peroxidase.

electron donors or at low peroxide concentrations, the product of the oxidation reaction (Compound I) decomposes slowly (Pruitt et al, 1990). In the presence of one-electron donors (including most of the chromophores used in peroxidase assays), Compound I is reduced in sequential, one electron transfer reactions to produce Compound II, i.e., organic free radicals, native enzyme and the oxidized donor. Therefore, the original state is restored by two successive reductive steps of Compound I by a hydrogen donor. Most assays (spectrophotometric) measure the appearance of oxidized donor. (See Section 1.2.7, below). An excess of hydrogen peroxide will inactivate the enzyme. The structure of the enzyme-substrate compounds of peroxidase have been determined by X-ray absorption spectroscopy (Chance et al, 1984). The valence states of the various compounds were also determined. The mechanism of horseradish peroxidase was elucidated by analysis of the structural changes observed in the reaction intermediates. A similar study on the oxidation - reduction potentials of the compound intermediates in the HRP reaction was undertaken by Hayashi & Yamazaki (1979). Attempts to identify the possible structure of the iron atom of compound II were performed. Techniques used to study the compounds included NMR, ESR, magnetic circular dichroism and others. A red or green (compound III and IV, respectively) colour will appear if there is substrate inhibition. There is a narrow optimum concentration range for

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hydrogen peroxide. If too low a concentration of hydrogen peroxide is used, there is decreased activity and too high a peroxide concentration brings about substrate inhibition. Cyanide and sulphide reversibly inhibit HRP but carbon monoxide does not. HRP is quite sensitive to bacteria, bacteriostatic agents and other chemicals found in tap water. So, it is recommended that ultrapure water be used to wash all glassware and in preparation of all reagents to be used in connection with HRP (Tijssen, 1985). HRP may be inactivated by polystyrene plates if Tween 20 is omitted (Berkowitz & Webert, 1981).

Detailed studies on the complete amino acid sequence of horseradish peroxidase isoenzyme C were presented in 1979 by Welinder. Previous to this the amino acid sequence of various peptides isolated from HRP had been studied (Welinder, 1973, Welinder & Mazza, 1977). Tryptic and thermolysin digested peptides that accounted for all the amino acid residues of the horseradish peroxidase peptide chain had been isolated. The full physical and chemical interpretation of amino acid sequence data could not be made as there was a lack of information about what amino acid residues were located proximally and distally to the porphyrin moiety of HRP. Work on the elucidation of the primary structure of HRP was hampered by the fact that successive electrophoretic steps used to isolate tryptic digestion peptides resulted in a complete loss of the large disulphide bridged peptides. In 1973, a histidine-positive disulphide bridged peptide was isolated, that had previously been lost in the electrophoretic steps. It was discovered that this peptide sequence is positioned distally to the haem. This sequence may be homologous to that of the distal histidine sequence of the globin family and also might play a part in the active site of horseradish peroxidase. Turnip peroxidases were also sequenced by Welinder. When these five peroxidases were characterized by peptide mapping two sequences were present that were highly homologous between four turnip peroxidases and horseradish peroxidase isoenzyme C. The homologous sequences both contained histidine. Further studies on peroxidases by Welinder & Mazza (1977) involved the sequencing of amino acids around the histidine residues of four turnip peroxidases. Twenty five residues around the histidine proximal haem and thirty four residues around the (probably) distally located histidine were sequenced. These sequences

were compared with histidine-containing sequences of horseradish peroxidase isoenzyme C. All these plant peroxidases contain highly homologous histidine-containing sequences, located near the peroxidase haem prosthetic group. It appears that these sequences are conserved in plant peroxidases. Over a number of years, Welinder pieced together the horseradish peroxidase isoenzyme C amino acid sequence and studied homologous and conserved regions between this peroxidase and other proteins, specifically other peroxidases and globins. Other haemoproteins studied, that contain histidine sequences close to the haem prosthetic group, include globins and cytochrome c peroxidase. In 1991 Welinder reported on the study of structural and other properties of plant, fungal and bacterial peroxidases (Welinder 1991). Peroxidases from plants, fungi (including yeast) and bacteria belong to the Plant Peroxidase Superfamily. A property that peroxidases from this family possess is similar protein folding. Animal peroxidases are structurally unrelated and constitute the Animal Peroxidase Superfamily. Yeast and plant peroxidases are similar in reaction mechanisms and mode of ligand binding. The reaction with aromatic donors is unique to plant peroxidases. Several conserved residues and structures have been found in all plant fungal and bacterial peroxidases.

1.2.5.   Stability Properties:

Thermal inactivation kinetics of horseradish peroxidase have been studied in some detail. Horseradish peroxidase is one of the most heat stable enzyme in vegetables and has high inherent thermostability. Peroxidase causes unfavourable colour and off-flavour during food preservation processes (Chang et al, 1988). The level of activity of peroxidase indicates the effectiveness of blanching treatments in food processing and this is one of the reasons why the thermal inactivation of horseradish peroxidase is of interest. Chang et al (1988) examined the thermal inactivation kinetics of HRP in the presence of sugars by Differential Scanning Calorimetry (DSC) and by determination of residual activity. This study was performed so that the reaction order for thermal decay of HRP could be evaluated and to distinguish between thermal denaturation and loss of enzyme activity. Generally, a deviation from first-order kinetics is seen for the residual decay curve of HRP. This deviation

has been explained by several mechanisms, including the formation of enzyme aggregates with different heat stabilities, presence of heat-stable and -labile isoenzymes and series-type enzyme inactivation kinetics. Chang and co-workers found that the thermoinactivation of peroxidase does not follow first order kinetics. Both DSC and residual activity determinations showed that the apparent reaction order for thermoinactivation was 1.5. The presence of isoenzymes of different thermal resistance were thought to be responsible for the non-first order kinetics. Electrophoresis and isoelectric focusing revealed seven or more proteins from the original "peroxidase". Four of these proteins were peroxidase isoenzymes. The 1.5 order of thermal inactivation could then be accounted for because of the presence of heat-stable and heat -labile isoenzymes, i.e., as a result of the heterogeneity of the HRP solution studied. The effect of sucrose, a non-reducing sugar, on the thermal stability of peroxidase was studied by DSC. The addition of sucrose resulted in an increase in the maximum denaturation temperature, as sucrose concentration increased. This suggested that the addition of sucrose stabilized the enzyme against thermal denaturation. At low sucrose concentrations, as revealed by the determination of residual activity, there was in fact a decrease in thermal stability of HRP. Other sugars, i.e., reducing sugars such as fructose, glucose and lactose incubated with HRP brought about a more rapid inactivation of HRP than did the presence of sucrose.

Ugarova et al (1979) investigated the thermostability of horseradish peroxidase that had been chemically modified at the ε-amino groups of lysines with carboxylic acid anhydrides and picryl sulphonic acid. An increase in the thermostability of the modified enzyme was due to a decrease in conformational mobility in the protein moiety around the haem. It was the degree of modification, i.e., the number of modified ε-amino groups of lysines, rather than the nature of the modifier that was important. Modification of the enzyme resulted in restricted conformational mobility, as seen from circular dichroism (CD) spectra. The reduced flexibility, seen at four of the six lysines of horseradish peroxidase, was correlated to enhanced stability. However, the modification of all six lysines, which resulted in reduced conformational flexibility of the protein in the vicinity of the haem, resulted in

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reduced thermostability. The effect of modification can be taken beyond its optimum and over-rigidification of the protein can occur that does not confer enhanced stability. This implied that there was a distinct correlation between the conformational changes and the change in thermostability of the enzyme after modification of its functional groups.

Techniques such as enzyme immobilization, protein engineering and other physical and chemical modification procedures can affect the thermostability of an enzyme. Weng et al (1991) studied the thermal stability of immobilized peroxidase and compared the stability with native, soluble peroxidase. Inactivation curves for soluble peroxidase showed biphasic behaviour. Two theories exist to explain this phenomenon. The first theory is the two fraction theory in which a heat-labile and a heat-stable fraction exist, as described by Chang et al (1988). The second theory puts forward the idea that an intermediate, that is partially denatured, is formed during the heating process. This intermediate, or partially denatured protein that shows peroxidase activity, has a higher thermal stability than the native enzyme. With peroxidase that is covalently immobilized on glass beads, biphasic behaviour was observed for temperatures below 80°C. At temperatures above 80°C the inactivation followed first order kinetics. The heat inactivation of peroxidase immobilized on glass beads was monitored in a variety of organic solvents. This was of particular interest as immobilized enzymes may be used in organic solvents in applications such as thermal processing/bioindicators.

Horseradish peroxidase is a metalloprotein in which calcium contributes to the structural stability of the protein (Haschke & Friedhoff, 1978). Calcium has a role in the maintenance of the molecular conformation of HRP. Bound calcium may be removed by incubation of the peroxidase with guanidine hydrochloride and EDTA. Thermal stabilities of native and calcium-free enzyme were studied. Calcium removal decreases the thermal stability of the enzyme. This indicates that the Ca^{2+} ions function in maintaining the protein conformation of the enzyme. The function of calcium in a number of metalloproteins is to stabilize the enzyme structure.

1.2.6.   Recombinant Horseradish Peroxidase:

A synthetic gene encoding horseradish peroxidase isoenzyme C has been synthesized and expressed in *Escherichia coli* by Smith et al (1990). The gene that was constructed was based on the amino acid sequence of the mature protein, as described by Welinder (1979). A nonglycosylated recombinant enzyme was produced in an insoluble inactive form. Insoluble products frequently result when heterologous proteins are expressed in *E. coli*. The HRP C produced was solubilized and active enzyme was obtained when specific folding conditions were used. The reduced, denaturant-solubilized polypeptide was folded in the presence of calcium ions and haem to give active enzyme. Specific concentrations of urea, Ca^{2+} and haem were used. However, purification of active recombinant HRP C yielded about half the activity of native HRP C, when assayed under similar conditions. It appears that glycosylation is not essential for correct folding and activity. The N and C termini are not encoded for in the construction of the gene. Neither terminus is required for enzyme folding or activity. Calcium ions, as discussed by Haschke & Friedhoff (1978), are important in the folding and structure of the enzyme. The binding of calcium ions is an obligatory step in folding before correct disulphide bridge formation and haem incorporation can be completed. Peroxidase is the only haemoprotein in which calcium ions have been reported as a constituent. In the future, site directed mutagenesis may be used to identify calcium ion binding regions. There may be homology between these calcium binding residues and cytochrome *c* peroxidase. It is hoped that the use of nonglycosylated recombinant HRP C may yield crystals suitable for X-ray crystallographic analysis so that the much awaited three-dimensional structure of HRP may be elucidated.

1.2.7.   Horseradish Peroxidase Catalyzed Reactions:

Peroxidases are enzymes that catalyze the oxidation of various substrates. Horseradish peroxidase decomposes two molecules of hydrogen peroxide, the natural substrate into water and oxygen by a two-electron oxidation step. Thus, hydrogen peroxide is reduced in the presence of a hydrogen donor. The specificity that HRP

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has for the second molecule of hydrogen peroxide is low and many other electron donors may be used (Conyers & Kidwell, 1991). The native enzyme is regenerated by electron transfer from a hydrogen donor, which is oxidized. This is a redox or oxidation/reduction type reaction (Frew et al, 1986). Monitoring of the oxidized donor gives an indirect means of monitoring the concentration of hydrogen peroxide involved in the reaction.

Hydrogen peroxide is the natural substrate. Indicator molecules, that can be used to monitor activity, are often referred to as substrates. Substrates used to demonstrate the presence of peroxidase activity are numerous, widely available and are well characterized. Substrates that are involved in colorimetric, luminescent, fluorimetric, electrochemical and hydroxylation reactions are all used to detect HRP activity and to quantify hydrogen peroxide. The detection and quantitative determination of hydrogen peroxide is of importance in many areas such as industrial and clinical applications. Hydrogen peroxide is also an analyte that is of environmental interest, as it plays a key role in the atmospheric oxidation of SO_2 to H_2SO_4. In this case very low levels of hydrogen peroxide are monitored in hydrometers (Genfa & Dasgupta, 1992). The presence of peroxidase activity is widely used as a detection step in immunoassay and histochemical and immunoblotting procedures. Because of the wide use of HRP and the wide need for the detection of H_2O_2, a large number of procedures have been developed for these purposes. Detection methods include spectrophotometric, chemiluminescent, fluorimetric and electrochemical, using the substrates previously mentioned. All these techniques have relative merits. Recently, a fluorimetric assay system that utilizes haematin as a peroxidase substitute in hydrogen peroxide determinations has been reported (Genfa & Dasgupta, 1992).

1.2.7.1.   Colorimetric Assays:

Horseradish peroxidase combines with hydrogen peroxide and is reduced in two successive steps by a hydrogen donor. This is a redox type reaction, as both oxidation and reduction take place. Many chromogenic substrates act as hydrogen

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donors; on oxidation these compounds form a coloured product. The appearance of the product can be monitored spectrophotometrically, typically using a microplate reader. Chromogenic substrates for HRP include o-phenylene diamine (OPD), 2,2'-azino-di-[3-ethyl benzthiazoline-sulphonate] (ABTS) and 3,3',5,5'-tetramethylbenzidine (TMB) (Madersbacher & Berger, 1991). 4-Chloro-1-naphtol (4-CN), 3,3'-diaminobenzidine (DAB) and 3-methyl-2-benzothiazoline hydrazone hydrochloride (MBTH) are also used (Conyers & Kidwell, 1991). Chromogenic peroxidase substrates are often divided into two categories, depending on whether they form soluble or insoluble products. Solubility of the product must be considered when a chromogen is being chosen for a particular application. The colour produced by the reaction must also be considered. A chromogenic compound used in a detection step should be relatively inexpensive, easy to use, soluble, non-carcinogenic and otherwise safe to handle. The product should also be safe, stable and should exhibit high molar absorptivity (Gerber et al, 1985). Some chromogens for HRP are carcinogenic and others lack sensitivity. Many considerations must be made when choosing a chromogen and assay for a particular application.

1.2.7.2.   Chemiluminescent and Fluorimetric Assays:

Chemiluminescence is another reaction type catalyzed by HRP participates. Chemiluminescence constitutes light-emission occurring during a chemical reaction. The most commonly used chemiluminescent reagents are luminol and related hydrazides (Coulet & Blum, 1992). Horseradish peroxidase can oxidize luminol in an alkaline solution in the presence of hydrogen peroxide. The products of the reaction are 3-aminophthalate and light (Hool & Nieman, 1988). In a bienzyme-type system, the amount of hydrogen peroxide that is produced by an oxidase may be assayed by using excess HRP and luminol in the reaction mixture. The steady-state chemiluminescence intensity can be measured at 425nm. Hydrogen peroxide can be detected at very low levels using this system. Bacterial and firefly luciferase operate in bioluminescent systems. These luciferase bioluminescent enzymatic systems and the chemiluminescent H_2O_2/peroxidase/luminol systems offer the means of analyzing

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the majority of analytes and enzymes of clinical interest (Roda et al, 1991). Immobilized luminol chemiluminescence reagent systems can be used to determine hydrogen peroxide in flowing stream/bioreactor situations. Enhancers such as luciferin, *p*-iodophenol or *p*-hydroxy cinnamic acid are used to increase light emission from a flash signal to a steady-state output. Chemiluminescent techniques offer greater sensitivity than other procedures.

Hydrogen peroxide can be detected by a fluorimetric assay. Hydrogen peroxide reacts with HRP and *p*-hydroxy-phenylacetic acid (POPHA), to form the fluorescent dimer of POPHA. Fluorescent detection techniques are employed to quantify the dimer, the amount of which is proportional to the concentration of hydrogen peroxide involved (Lazrus et al, 1985). The peak excitation wavelength of the fluorescent dimer produced is 320nm and the emission wavelength is 400nm. An automated version of this assay can be used to determine hydrogen peroxide in environmental aqueous solutions and precipitations. This assay can also be used to detect peroxidase activity.

1.2.7.3. Electrochemical Reactions:

Electrochemical reactions involving HRP utilize a hydrogen donor that can be monitored voltametrically upon reduction. The reduction of the oxidized donor can be monitored and therefore the concentration of hydrogen peroxide involved in the reaction can be monitored. A hydrogen donor that can act as an electron mediator and that can be followed voltametrically upon reduction is essential (Sanchez et al, 1990). Electron mediators that act as hydrogen donors that can be used for the peroxidase-catalyzed reduction of H_2O_2 include hydroquinone, o-toluidine, resorcinol and catechol. Mediator/redox electrodes employing HRP will be discussed in Section 1.4.

1.2.7.4. Other Reactions involving Horseradish Peroxidase:

Horseradish peroxidase can function as a catalyst in a number of water-immiscible

organic solvents. Most enzymes can act as catalysts in nearly anhydrous organic solvents. This is because the water essential for enzymatic activity is tightly bound to the enzyme molecules and may remain bound even when the bulk water is replaced by organic solvents. Enzymatic function in organic solvents allows the determination and quantification of analytes that are not soluble in water/buffer systems (Kazandjian et al, 1986). These include cholesterol, that can be determined by utilizing a bienzyme system involving cholesterol oxidase and HRP.

The reactions catalyzed by HRP in organic solvents include hydroxylations, N-demethylations, sulphoxidations and other oxidations of various organic substances (Urrutigoity & Souppe, 1989). Modification of HRP, as discussed in Section 1.3, can render the enzyme more active and soluble in organic solvents. Donors used for HRP catalysis in conjunction with hydrogen peroxide include p-anisidine and 9-oxoellipticine.

Peroxidases can catalyze one-electron oxidations of phenols. Phenols that can be acted on by HRP include p-hydroxyphenylacetate and p-cresol. This results in the formation of phenoxy radicals, which spontaneously couple to form polymers (Popp et al, 1991). The enzyme-catalyzed phenoxy radical polymerization is favoured over other polymerization reactions used to oxidize phenols. Lignin is a phenolic resin and p-cresol can be incorporated into this via a peroxidase-catalyzed copolymerization in nonaqueous media.

Horseradish peroxidase can catalyze the hydroxylation of some aromatic compounds by molecular oxygen in the presence of dihydroxyfumaric acid as the hydrogen donor (Klibanov et al, 1981). HRP can catalyze the oxidation of 9-methoxyellipticine to 9-oxoellipticine in the presence of H_2O_2. This reaction can be performed in diethylether if an enzyme modified with dithioesters is used (Souppe et al, 1988).

1.2.8.   Applications of Horseradish Peroxidase:

Horseradish peroxidase is widely used in enzyme assays, immunoassays, immunohistochemical procedures and in bienzyme systems which generate hydrogen peroxide. Tijssen (1985) details the properties of HRP that make it suitable as an enzyme-label in enzyme linked immunosorbent assays (ELISA). No enzyme fulfills all the criteria for an ideal label in enzyme immunoassays, but HRP possesses several properties that make it suitable as an enzyme label. These include high turnover number, stability upon storage and stability when used under various different assay conditions, i.e., variations in pH, ionic strength, buffer types and temperatures. There are few HRP inhibitors and these are not usually present as interfering substances in samples to be assayed, such as blood and urine. Also, HRP is relatively cheap and available in pure form. It is well suited for the preparation of enzyme conjugated antibodies due to its excellent stability characteristics and ability to yield chromogenic products. It is easily detected using a wide range of substrates by colorimetric, fluorimetric, luminescent and various other assays. HRP is highly specific and sensitive. For enzyme immunoassays (EIA), spectrophotometric assays using colorimetric substrates for the HRP enzyme label are generally employed. Closely related analytes can be distinguished by antibodies and it is this fact that is exploited in EIA. Antibodies can be used to quantify the amount of antigen in a sample, with a high degree of accuracy. One type of immunoassay is enzyme-linked immunosorbent assay (ELISA). The antibody of interest is immobilized onto a solid support, sample is added, unbound sample is removed by washing, the second antibody (specific for a different site to the first antibody) is added and finally, there is a detection step. The second antibody is labelled with an enzyme (Stryer, 1988). Less than a nanogram of protein can be detected by the rapid and convenient ELISA. The amount of second antibody that binds is proportional to the quantity of antigen of interest in the sample. The enzyme-second antibody conjugate can convert an added colourless substrate into a coloured product, or a nonfluorescent substrate into an intensely fluorescent product. The amount of product formed will be proportional to the amount of antigen. This type of sandwich immunoassay is a widely used technique.

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Conjugation of antibody to enzyme label must be easily accomplished. Also, enzyme - antibody conjugates produced must be active and stable. Nakane & Kawaoi (1974) used sodium periodate to conjugate HRP to antibodies. In this procedure the carbohydrate moiety on HRP is oxidized by periodate. Aldehyde groups are produced and these form Schiff bases with nonprotonated amino groups of the antibody. This method has been modified and improved so that highly active conjugates are obtained with high recovery (Tijssen & Kurstak, 1984). Horseradish peroxidase contains six lysine groups and these are often targeted in conjugation procedures using crosslinking reagents. The strategy and rationale for the use of crosslinking (homo- and hetero-bifunctional) reagents in enzyme - antibody conjugate preparation is the same as for their use in protein modification studies and for their use in production of immunogens. The use of heterobifunctional crosslinkers, i.e., containing two different reactive groups, is a preferred method of conjugate production as the protein and antibody of interest may be reacted in a step-wise manner. This reduces the occurrence of unwanted side reactions. Avrameas & Ternynck (1971) prepared peroxidase-labelled antibodies by crosslinking with glutaraldehyde. This technique has been used, updated and improved extensively. Dimaleimides were used by Weston et al (1980) to prepare peroxidase-immunoglobulin conjugates. Nilsson et al (1981) conjugated HRP and immunoglobulins using a heterobifunctional reagent, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). The above are the most commonly used conjugation procedures. These protocols can be adapted to suit the specific needs of the antibody, or other components in question. Many other conjugation procedures exist for the preparation of enzyme - antibody conjugates (Tijssen, 1985, Peeters et al, 1989).

Horseradish peroxidase is also used in immunohistochemical and immunoblotting procedures. Enzyme labelled reagents used in immunohistochemical and immunoblotting procedures are detected using soluble chromogenic substrates which precipitate following enzyme action. When they precipitate, these substrates leave an insoluble coloured product at the site of bound enzyme. Antibodies can be raised to specific molecules, for example, proteins from brain or other organs. The exact

functional location *in vivo* of a particular molecule can be located and visualized by this procedure of immunolocalization. Horseradish peroxidase neurohistochemistry is one of the most frequent methods used for tracing neuronal connectivity within the central nervous system (Mesulam, 1978). This technique involves the tracing of neural connections after the injection of HRP (Olucha et al, 1985). HRP has proven to be valuable for demonstrating uptake and retrograde axonal transport of exogeneous proteins by neurons (Bunt et al, 1976). In this procedure HRP is injected into the test specimen. After a specific period of time tissue slices from the brains of specimens are fixed. A particular chromogen is used to stain for peroxidase activity. The reaction product formed is usually insoluble granules. A study of the sites at which HRP activity is visualized can be used to give details about what nerves of the cortex and other parts of the brain transport HRP, and therefore proteins. Tetramethylbenzidine (TMB), a chromogenic substrate for HRP, is often used in these histochemical applications. (TMB and other substrates of HRP are discussed in Section 1.2.7). Another use of HRP in physiological studies is in the study of fluid pinocytosis (Oliver et al, 1984), the uptake of media by enclosures in small membrane vesicles that bud from the cell surface. This process, either of fluid or adsorptive pinocytosis, occurs in nearly all cells. Fluid pinocytosis can be observed and quantified by using HRP as a marker since it is readily soluble, membrane-impermeable and does not alter cellular activities. Also, HRP is not metabolized within the cell, has no binding affinity for the plasma membrane and can be readily visualized microscopically.

Bienzyme systems often incorporate the use of HRP. The schematic representation of a bienzyme system is shown in Fig. 1.3.

enzyme 1   enzyme 2

Substrate $\Longrightarrow$ **Product 1** $\Longrightarrow$ **Product 2**

Fig. 1.3.   Bienzyme System.

The use of bienzyme systems is incorporated in many bioassays that do not directly produce a detectable product. Immobilization of these systems onto an electrode is frequently seen in biosensors. HRP is often used as enzyme 2 to convert a product from enzyme 1 into a detectable form.

Horseradish peroxidase is commonly used as a model enzyme for protein modification studies. Protein modification strategies will be discussed in Section 1.3. Modification has varying effects on enzyme reactivity. Changes in thermal stability, resistance to denaturing agents, changes in kinetic parameters and alterations in activity in nonaqueous organic media may occur. Protein modification involving the specific use HRP is discussed in Chapter 4.

1.3. PROTEIN MODIFICATION:

1.3.1. Protein Stability:

Recent advances in molecular biology have enabled the production of large quantities of active recombinant proteins. These can be used in industrial and medical applications, such as therapeutics, diagnostics, bioreactors, fine chemicals, immobilized enzymes, enzyme electrodes and biosensors. Proteins used in biotechnology must be able to function reliably over a long period of time. Stable proteins and enzymes are essential to broad biotechnological applications. Studies on thermostable proteins isolated from thermophilic organisms and on the denaturation of enzymes at different temperatures, together with investigations of enzyme function in organic solvents, have given a general understanding of inherent protein stability. Strategies for enhancing stability are being developed.

The activity of a protein requires proper folding, to ensure that structural and functional integrity of the active domain are retained. The three-dimensional structure of the enzyme is in a functional active form in a native enzyme. However, the three-dimensional structure and hence the integrity of the enzyme (or other

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functional protein) can be irreversibly disrupted by a range of forces. These include physical forces such as heating, freezing and irradiation, chemical forces such as oxidation, reduction, ionic strength, extremes of pH, effects due to chemicals, solvents and metal ions and biological forces such as enzyme modification and degradation. Heat and oxidation are the most common forms of structural disruption. Industrial processes frequently involve elevated temperatures and the use of chemicals and organic solvents. The forces mentioned above may not be encountered by a protein *in vivo*. A protein needs to maintain its functional characteristics *in situ* in an industrial application. So, when a protein is used in a novel situation, problems with stability may arise. Enzymes can, however, be stabilized by a variety of procedures to ensure that activity and function are not lost. Stabilization of these proteins can be achieved through changes in the protein through mutagenesis or by either covalent or non-covalent chemical modification of the surface chemistry of the protein with specific reagents and also with antibodies (Shami et al, 1989). Proteins can be deliberately stabilized by a number of other methods including immobilization and use of solutes and additives (O'Fagain & O'Kennedy, 1991). Details of these techniques will be given in this section.

As mentioned, physical, chemical and biological forces may lead to denaturation of a protein. Stability in a folded protein is a balance between the stabilizing (mostly hydrophobic) interactions, and the tendency towards destabilization that is caused due to the loss of conformational entropy as the protein adopts the unfolded form (Nosoh & Sekiguchi, 1990). An enzyme in solution undergoes flexing and minor conformational changes, for example, during substrate binding and catalysis. Within limits these conformational changes are reversible. Protein denaturation is the phenomenon that occurs as stability is lost. Conformational stability relates to the free energy change under defined conditions for the reaction N $\leftrightarrow$ U, where N is native folded protein and U is denatured unfolded protein (O'Fagain & O'Kennedy, 1991). U is changed to X in irreversible inactivation (U $\rightarrow$ X), where X is inactivated protein (Nosoh & Sekiguchi, 1990). Overall, denaturation refers to conformational changes that lead to unfolding of the protein backbone and therefore loss of molecular function. This may or may not be reversible. Inactivation results

in changes in the degree of association or aggregation of the molecules or from covalent changes in the protein backbone, together with primary structural changes, such as peptide bond hydrolysis or destruction of amino acid side-chains. Activity is lost when the unfolding disrupts the integrity of the molecule's active or functional site(s). At temperatures above 50°C, enhanced thermostability implies a shift in the conformational equilibrium towards the native form of the enzyme (Stellwagen, 1984). When an enzyme is heated a number of molecular events begin to take place. The enzyme molecule partly unfolds due to disruption of noncovalent interactions that maintain the catalytically active conformation. This is reversible when an enzyme molecule is cooled. Reactivation may take several hours and may require quick or slow cooling or prolonged incubation at intermediate temperatures. Denatured enzymes may become partially or fully reactivated only after many hours of incubation under normal conditions. If heating persists, activity will not return on cooling as irreversible inactivation processes will have taken place (Ahern & Klibanov, 1985, Mozhaev & Martinek, 1982).

The activity of most enzymes continuously decreases over time. A knowledge of enzyme deactivation phenomena is needed if enzymes are to be used in applied situations. Different chemical reactions follow different kinetic orders of reaction. The loss of biological function, by denaturation/inactivation of an enzyme, is the result of a chemical or biological reaction and should be capable of description by a particular kinetic order equation. The reaction orders are as follows: first-order, second-order, pseudo first-order and zero-order. In a zero-order reaction, the reaction rate does not depend on the concentrations or activities of the reactants. The reaction rate is dependent on one reactant concentration only in the case of a first-order reaction. A second-order reaction rate depends on the concentrations of two reactants and a pseudo first-order process is one in which two reactants are involved, but only one of these reactants appears to change during the reaction (O'Fagain et al, 1991b). Activity loss with time of an enzyme at a particular temperature can be studied to determine what type of order the decay rate follows. Data, in the form of the percentage of activity versus time, can be fitted to various exponential rate equations using a dedicated computer programme. Visual observation of the graphic

fit and comparison of chi squared values for each equation and set of data will reveal which order the denaturation follows. However, thermal deactivation does not always occur as simple exponential decay (Gianfreda et al, 1984). Non-first order exponential decay may appear as two-slope logarithm activity versus time curves. Or, a time progressive increase in the rate of deactivation may be visible, resulting in constant enzyme activity for an initial time period, followed by a fast decrease in the rate of enzymatic reaction. Enzyme denaturation curves can involve a series-type mechanism (Henley & Sadana, 1985). An active enzyme precursor and a final enzyme state with possible non-zero activity was proposed to categorize enzyme deactivation curves. The deactivation curves may be broadly classified into two major categories. In the first category, the activity seen in the thermal deactivation pattern is always less than the initial activity. In the second category, the activity may be more than the initial activity for some time period.

If the loss of activity of a particular enzyme does not follow one of these distinct orders, then this is probably due to series-type mechanisms discussed by Henley & Sadana (1985) and Gianfreda et al (1985) or to the presence of two isoenzymes in the original enzyme preparation (Chang et al, 1988). Knowledge of an enzyme's inactivation kinetics is useful when designing experiments to further assess the stability of an enzyme (following denaturation studies). Immobilized enzymes may show different kinetic values relative to the soluble, native enzyme. Function in organic solvents and in the presence of various sugars may alter the kinetic pattern and increase the maximum denaturation temperature of an enzyme (Weng et al, 1988). Accelerated storage studies can be used to predict the useful shelflife of a chemically modified enzyme at a temperature of interest. This is of use for enzyme derivatives that may be used in clinical applications (O'Fagain & O'Kennedy, 1991).

1.3.2.   Production of Stabilized Proteins:

An enzyme may lose its activity by a variety of mechanisms, as discussed above. Elevated temperatures, or adverse conditions such as freezing, irradiation, oxidation

and other chemical, physical and biological forces mentioned previously, will bring about this denaturation. Many microorganisms, however, are capable of living at elevated temperatures and therefore, their enzymes are capable of functioning at elevated temperatures. Thermophilic organisms are described as those capable of growth at temperatures ranging from 55°C to 100°C (Brock, 1985). Bacteria with temperature optima in the range of 65°C to 105°C have been obtained in pure culture. The upper temperature for life in liquid water is somewhere between 110°C and 200°C. Peptide bonds (in proteins) and phosphodiester bonds (in nucleotides) and amino acid residues are destroyed at temperatures above 250°C. The macromolecules of thermophiles are inherently more stable than those of other organisms. Thermophiles are the traditional source of stable enzymes. A study on the stability of molecules from thermophiles can give knowledge as to what strategies should be developed for enhancement of molecular stability. Propylamine transferase from *Sulfolobus solfataricus* retains its quaternary structure in the temperature range 25 - 85°C (Facchiano et al, 1992, Ragone et al, 1992). This archaebacterium is an extreme thermophile with an optimum living condition at 90°C. There is a conformational transition in enzyme structure that occurs at 45°C. Two different activation-energy-dependent processes occur, one at a temperature higher than 45°C and one at a temperature lower than 45°C. In this way, thermal stabilization is driven by a conformational equilibrium between two forms of different stability. There are two enzyme forms present in solution that are characterized by different catalytic properties. Temperature modulation of the transition between two protein populations at equilibrium appears to operate. Hydrophobic interactions arising from burying of the accessible surface residues and conformational entropy are the two major factors that contribute to stability. The thermal stabilization of the enzyme depends on effects related to both an overall increase of flexibility and a decrease in the area buried upon folding. These studies give a model that can be used to investigate protein stability. A highly thermostable α-amylase has been isolated from *Bacillus stearothermophilus* (Brosnan et al, 1992). The half lives of irreversible thermoinactivation of this enzyme are 1.9 min. and 12.5 min. at pH 5.0 and pH 8.0, respectively. An investigation into the causes of irreversible thermoinactivation reveals the molecular mechanisms responsible for

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inactivation. Heat-induced unfolding of the molecule followed by re-folding into incorrect structures causes irreversible thermoinactivation. The monomolecular conformational scrambling occurs due to breakdown in non-covalent interactions responsible for maintaining the protein in a tightly coiled catalytically active conformation. Hydrophobic interactions were shown to be the most important non-covalent mechanisms involved in the process. Electrostatic interactions, including hydrogen bonding, were also shown to be involved.

Thermostability can result from only small amino acid sequence changes. Enhanced intrinsic stability requires only minute local structural changes, so general strategies of stabilization are hard to establish (Jaenicke, 1991). Despite this, the effects of amino acid substitutions can be predicted to some extent. Conformational and other changes must also be taken into consideration, however (Nosoh & Sekiguchi, 1990). Amino acid substitutions can lead to increased thermostability of a modified protein. Frequently, increased salt bridge formation results in increased thermostability. Generally, particular amino acid substitutions that bring about stabilization of one protein, may not be applicable to stabilization of another protein. Proline residues responsible for thermostability in an enzyme from *Bacillus thermoglucosidasius* occur with high frequency in the amino acid sequence (Watanabe et al, 1991). Most of the additional prolines occur in coils within the loops binding adjacent secondary structures. Comparing this enzyme to another, less stable, glucosidase it was noted that extra proline residues were responsible for the thermostability seen between the two different enzymes. Protein engineering can be used to explore the molecular basis of protein stability and also to modify protein stability. Protein engineering by site-directed mutagenesis can lead to production of stabilized proteins via specific mutations of amino acid sequence. New genes can be constructed that involve direct changes in amino acid sequence. Single point mutations can substitute one amino acid for another. The resulting protein can then be tested to determine if the change in amino acid composition has had any effect on stability. A single amino acid replacement may modify and enhance the stability of a protein.

The applications of enzymes in biotechnology are limitless, but a basic limitation of

enzymes is their degree of stability. The fact that enzymes find use in many biotechnological applications, such as bioreactors and biosensors, has meant that much research has gone into the enhancement of enzyme stability. The stability of an enzyme may be altered by a number of methods, including protein engineering (as mentioned above), immobilization (as discussed in the Section 1.4.3) and by chemical modification and use of organic solvents, discussed here.

1.3.3.   Chemical Modification and Its Application to Horseradish Peroxidase:

Chemical modification of enzymes can be used to study and stabilize proteins. It is one of the most useful methods of identifying the functional groups of a protein (Imoto & Yamada, 1989). Bifunctional reagents or crosslinking reagents are used to chemically modify and thereby stabilize protein molecular structure (Ji, 1983). They have also been used to investigate the spatial arrangements and functions of various molecular entities in multiprotein complexes and in identification of receptor binding sites (Kenny et al, 1979) and have been used to analyze the quaternary structure of complex enzymes and to study lipoprotein structure (Swaney, 1986). Bifunctional reagents can be used to prepare enzyme-antibody conjugates, in immobilization techniques and, specifically for this discussion, in protein crosslinking for enhancement of stability. The last use of chemical crosslinkers is considered in detail below.

Crosslinking reagents are usually bifunctional reagents, i.e., reagents with two reactive groups, that can be used to introduce both inter- and intra-molecular bridges in proteins (Han et al, 1984). The introduction of intramolecular crosslinks can be used to stabilize the tertiary structure of an enzyme. Bifunctional crosslinking reagents are either homobifunctional, in which both reactive groups are the same, or heterobifunctional, in which both the functional groups are different. Homobifunctional reagents include imidoesters and bismaleimides, that are amino- and thiol- group specific respectively. Heterobifunctional reagents include derivatives of N-hydroxysuccinimide that are specific for both amino and thiol groups. Means & Feeney (1990) and Ji (1983) give a comprehensive list of homo-

and hetero-bifunctional crosslinking reagents. Thiol and amino groups are frequently targeted. These groups are reactive and occur frequently in protein primary structures. Other groups or side chains that can be targeted include carboxyl groups on aspartyl and glutamyl residues, guanidino groups of arginine residues and imidazole groups of histidine residues. Relatively little structural information is required concerning the target protein, and this is an advantage over protein engineering by site-directed mutagenesis. Crosslinking experiments are usually simple to carry out and results may be obtained quickly (O'Fagain & O'Kennedy, 1991). It is advantageous if incorporation of the crosslinker can be monitored, for example, by spectrophotometric titration of target residues. It is important that amino acids and functional groups targeted are not involved in catalysis. Active site residues can be protected by substrates or reversible inhibitors. In general, it is advisable to choose a target residue that is distant from the active site and that is not buried within the folded protein structure.

Bifunctional reagents crosslink different parts of the enzyme molecule. The active conformation of the enzyme should be maintained in any crosslinking procedure. In effect, "braces" are placed across the molecule and so unfolding of the enzyme is prevented (O'Fagain et al, 1988). It is known that unfolding is an essential step in protein denaturation (Torchilin et al, 1978) and this may be prevented by crosslinking. Molecular rigidification is achieved by crosslinking due to reaction of both functional groups of the bifunctional reagent with the enzyme backbone. A crosslinked enzyme should be more resistant to denaturation, by heat and other forces, than an uncrosslinked enzyme.

Stabilization effects may not be very dramatic and sometimes the effects seen may be due to chemical modification of the enzyme only by single point mutations as opposed to genuine crosslinking. Success or failure in increasing the thermostability of an enzyme by treatment with a bifunctional reagent largely depends on the length of the bifunctional molecule, and hence on the distance between the molecular centres to be crosslinked (Torchilin et al, 1978). Thus, a crosslinking reagent may form molecular bridges in one type of enzyme molecule and not in another. When

successful crosslinking occurs, it implies that the targeted enzyme has molecular distances that suited the crosslinking reagent employed. The modification of α-chymotrypsin by Torchilin et al (1978) was successful for some diamines used, but not for other diamines of different molecular length. Torchilin and coworkers (1979) used equal amounts of dithiols of HS-$(CH_2)_n$-SH (where n ranges from 4 to 10), i.e., molecules of the same type but with different lengths, to crosslink α-chymotrypsin. It was found the "best" reagent will gradually be selected for the formation of crosslinks, and will replace the initially formed one-point modifications, formed by almost all of the reagents used. This study also revealed intramolecular crosslinking renders the enzyme more stable against a wide spectrum of denaturing influences such as temperature, salt action and denaturing agents. This is useful if a modified enzyme is to be considered in an industrial application, as resistance to several actions is advantageous over resistance to just one.

The use of bifunctional crosslinking reagents is one method by which enzymes can be chemically modified. There are a variety of other methods. These are based on strengthening of hydrophobic interactions by nonpolar reagents and introduction of new polar or charged groups that give additional ionic or hydrogen bonds to the enzyme molecule (O'Fagain & O'Kennedy, 1991). Another chemical modification method is hydrophilization of the protein surface that reduces unfavourable surface hydrophobic contacts with water, as described in a recent report (Mozhaev et al, 1988). Tyrosine residues were nitrated and subsequently reduced to form aminotyrosines. Anhydrides and chloroanhydrides of aromatic carboxylic acids were used to introduce carboxylic groups onto α-chymotrypsin. The enzyme modified in this way was much more stable against irreversible thermoinactivation than the native enzyme (Mozhaev et al, 1988). The effects are due to hydrophilization leading to a reduction in the nonpolar surface area of the protein.

Additives that may be used to stabilize an enzyme include bivalent metal ions, ammonium sulphate, ethylene glycol, sucrose and various surfactants (Torchillin & Martinek, 1979). Enhanced enzyme stability can be seen when enzymes are coupled with large molecular weight polyhydroxy compounds, such as polyethylene glycol

(Imoto & Yamada, 1989). Enzyme modification can lead to enhancement of solubility and activity in organic solvents for some modified enzymes. Urrutigoity & Souppe (1989) prepared polyethylene glycol-bound HRP. This modified enzyme was soluble and active in chloroform and toluene. Wirth et al (1991) also coupled polyethylene glycol (PEG) to peroxidase, creating a modified peroxidase that was active in toluene, dioxane and dimethyl formamide.

Carbohydrate residues on HRP can be used as targets for conjugation of short aliphatic chains. Fourier Transform Infra-Red (FT-IR) vibrational spectroscopy is used to analyze the effect of such modifications (Arseguel et al, 1990(a)). The modification leads to an increase in the lipophilic properties of the enzyme. There was no adverse effect on the catalytic activity of the enzyme. The modified peroxidase dissolved more readily in organic solvents and at higher temperatures, than in aqueous media (Arseguel et al, 1990(b)). The use of horseradish peroxidase in industrial situations is limited by the hydrophobic nature of many of its substrates. HRP that can function in organic solvents in conjunction with organic soluble substrates has a much larger application span.

Immobilization-stabilization of trypsin by multipoint covalent attachment to agarose gels was performed by Blanco et al (1989) and optimization of this procedure, with respect to surface density of aldehyde groups, pH, reaction time and temperature was studied. Immobilization can be used as a method to stabilize an enzyme. Subsequent work by the same group on the stabilization of trypsin-agarose derivatives produced by multipoint covalent attachment revealed that borohydride reduction lead to stabilization of the derivatives. Optimization of the immobilization procedure is often necessary so maximum attachment and activity are obtained and so that minimum leaching occurs. The strategy of using several immobilization-stabilization techniques in conjunction with one another has been reported by Tor et al (1989). Horseradish peroxidase was one of the enzymes used in this study. Enzyme was coated with a low molecular weight polymeric glutaraldehyde. The glutaraldehyde layer was crosslinked by a second layer made of polyacrylamide derivatives. In this way a bilayered, chemically crosslinked, synthetic cage is

formed, surrounding the enzyme and increasing its stability to denaturation by rigidification of its structure. The "encaged" stabilized enzyme can be readily immobilized by crosslinking onto polyacrylamide-hydrazine gel. So, the enzyme is both stabilized and immobilized.

1.3.4. Screening for Modified Proteins:

Modified proteins can be purified by gel filtration and HPLC. Peptide mapping by reversed-phase HPLC can be used to identify modified residue(s) (Imoto & Yamada, 1989). Other methods for studying differences in native vs. modified protein include gel-electrophoresis, HPLC, fluorimetry, absorption spectra analysis, NMR, CD and FT-IR spectroscopy. Adsorptive voltammetry has been demonstrated to be a useful technique to assess the extent of chemical modification of an enzyme (Fernandez-Alvarez et al, 1990). Chapter 4 (Section 4.4.2.2) gives details on how a stabilized protein can be characterized.

1.3.5. Applications of Stabilized Enzymes:

Modified enzymes that show enhanced thermostability, are resistant to the action of denaturing forces and extremes of pH and that are active in organic solvents can be employed in similar applications to those mentioned for native HRP in Section 1.2.9. However, for modified HRP the applications are much broader and more diverse. These include use in biosensors, in clinical reagents and in various laboratory and industrial processes. Modified enzymes are more advantageous to use than native enzymes as higher temperatures and extremes of conditions can be used, without affecting the activity of the enzyme. Thus, modified enzymes can be used in novel situations.

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1.4.   BIOSENSORS:

1.4.1.   General Principles:

Horseradish peroxidase is often used as a coupling enzyme in bienzyme systems (See Section 1.2.8) and for this reason it has great potential for use in biosensors. A biosensor comprises a biochemically responsive material immobilized in close proximity to a suitable transducing element, designed to convert a biochemical response into an electrical response. The biocomponent may be an enzyme, lectin, antibody or antigen, microorganism, liposome, receptor, organelle or whole cell. The essence of a biosensor is two transducers. These two transducers relate the concentration of an analyte to a measurable electrical signal. The biochemical transducer converts the analyte into a chemical and/or physical response which is detected and converted into an electrical signal by the physical transducer (Stoecker & Yacynych, 1990). The strength of the signal is related to the concentration of the analyte. The schematic diagram of a biosensor is shown in Fig. 1.4. Using such devices a wide variety of analytes may be measured. The first type of biosensor, and that which all other biosensors are based on, was a Clark type sensor. The first biosensor to be commercialized was that of a glucose monitor produced by the Yellow Springs Instrument company (Lowe, 1984).
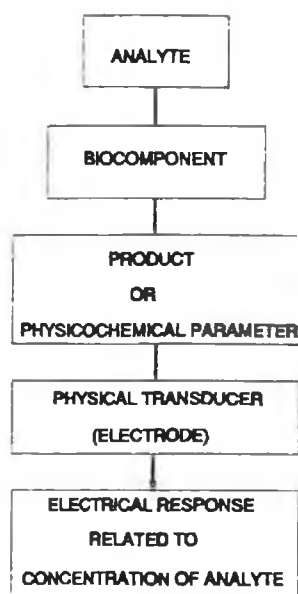
ANALYTE

BIOCOMPONENT

PRODUCT
OR
PHYSICOCHEMICAL PARAMETER

PHYSICAL TRANSDUCER
(ELECTRODE)

ELECTRICAL RESPONSE
RELATED TO
CONCENTRATION OF ANALYTE

Fig. 1.4.   Schematic diagram of a biosensor.

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Biosensors can take several forms including hand-held devices, laboratory instruments, flow type sensors (for large volumes) and implanted sensors for whole body monitoring (Gronow, 1984). Biosensors have evolved from the use of immobilized enzymes in conjunction with pH electrodes, amperometric devices and oxygen electrodes. Coughlan et al (1988) group analytical devices based on immobilized biocomponents into three classes. The first class comprises miscellaneous analytical devices, the simplest of which is a dipstick. Dipsticks consist of filter paper strips impregnated with an enzyme appropriate to the analyte to be measured, and a suitable chromogen. These dipsticks are commonly used by physicians and are usually disposable. They give an answer in very short periods of time. Visual comparison of colour produced relative to a standard chart gives an estimate of concentration of the analyte in question. Custom-designed, easy to use, basic spectrophotometers may be used to quantify the response. The most common dipstick is the one for glucose estimation in urine. Other devices in this group include enzyme brushes and visible immunodiagnostic assay kits. The second class of devices using immobilized biocomponents includes multisample autoanalysers and bioreactors. These bench-top type analysers use immobilized enzymes on membranes to measure starch sugars, such as glucose, ethanol and other substances. Bioreactors are flow devices that are used for on-line monitoring of processes in large volume production, such as food processing, fermentation and pollution monitoring. Biosensors are the third class of devices that use immobilized biocomponents, as described by Coughlan et al (1988). These biosensors are used in the determination of concentrations of particular analytes in biological fluids. Other areas of analytical biotechnology in which biosensors are used include veterinary and agricultural application, industrial processing and monitoring and in environmental and pollution control. Another class of biosensors are implantable biosensors or *in vivo* monitors on which work is being carried out. It is hoped that implantable biosensors will be used in providing information on key metabolites in the body, thereby directly delivering controlled amounts of drugs or other pharmaceuticals, as required, via an associated electromechanical drug dispenser or pump when necessary.

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Biochemical signals can be converted into quantifiable and processible electrical signals via a suitable transducing system. Biomolecules are specific and so a biomolecule specific for the analyte or species that is to be determined can be used to selectively recognize that analyte. When biological molecules interact specifically and reversibly there is a change in one or more physicochemical parameters (Lowe, 1984). The change in parameter could be a change in proton concentration, change in heat, absorbance, mass conductance or electron transfer or release or uptake of gases (O_2, CO_2, NH_3) or specific ions (NH_4^+, monovalent cations, CN^-, I^-). If these parameter changes are generated in close proximity to a suitable transducer, then they can be converted into electrical signals. Specific recognition can be realized by several types of biomolecules, including receptors, antibodies and enzymes. Antibodies and receptors have limited lifetime and can often only be used once. The complexing of antibody with antigen can take several hours. Because of these properties of antibodies, and also of receptors, these molecules are not as suitable as enzymes for use in biosensor construction (Renneberg et al, 1986). Enzymes have many properties that make them well suited for biosensors. Enzyme based biosensors have been most widely commercialized (Knight, 1989). The most desirable property of enzymes in relation to their use in biosensors is the fact that enzymes specifically recognize substrates, cosubstrates, cofactors, activators and inhibitors. Also, enzymes can be used thousands of times with the same efficiency. This efficiency can be stabilized and long life achieved when enzymes are immobilized onto the transducer in biosensor construction. As mentioned previously, biocatalysis generates a powerful physicochemical signal, that is electrode-detectable.

1.4.2.   Types of Transducers:

The transducer responds to the products of the biocatalytic process and relays this information to an interfacing detector. There are a wide range of physical transducers and one must be chosen to match the chemical or physical property, i.e., the physicochemical parameter, produced by the biocomponent. The transducer may take one of a number of forms including a metal or semiconductor electrode, an

electrochemical transducer, a transistor, a calorimetric transducer, a piezocrystal or opticoelectronic device (Stoecker & Yacynych, 1990). The transducer must be responsive to the compound of interest in the range required. The transducer must have a fast response time, be robust and reliable, be amenable to miniaturization and, in some applications, be amenable to sterilization. A practical transducer must be amenable to the immobilization and operation of the biological component in close proximity to it (Coughlan et al, 1988).

1.4.2.1.   Electrochemical Sensors:

Electrochemical transducers are the most popular type of transducers and most development work has gone into these. There are three main types of electrochemical sensors; potentiometric, amperometric and conductimetric (Janata, 1990), of which potentiometric and amperometric are the most common. Electrochemical sensors are widely used in analytical and clinical chemistry. The use of electrochemical detectors in biosensors has formed the basis for the biosensors that are available commercially. In a potentiometric sensor, local equilibrium is set up at the sensor interface and the electrode or membrane potential is measured. The potential difference generated at the sensing electrode is measured against an accurate reference electrode, under conditions of zero current flow (Coughlan et al, 1988). Essentially what is measured is potential difference, as opposed to an amperometric sensor, in which current is measured. The electrode potential in an amperometric biosensor is used to drive an electrode (redox) reaction and the current resulting from the reaction is measured (Albery et al, 1986). The potential is generated between the sensing electrode and an auxiliary electrode held at reference point. Potentiometric sensors require rapid electrode kinetics. With amperometric sensors, slower electrochemical reactions can be switched on by the electrode potential. Potentiometric sensors can be affected by large fluxes in voltage, but in these sensors there is no net consumption of analyte and so the overall analyte concentration is not affected. Therefore, the rate of mass transfer is unimportant. In an amperometric sensor, if mass transfer is not controlled, then the measured current can be inaccurate (Albery et al, 1986). In electrochemical sensors, sensing

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molecules are often coated onto and covalently bonded to the electrode (or probe) surface. A membrane may hold sensing molecules in place, excluding interfering species that may be present in the analyte solution. The sensing molecules react specifically with the substances to be detected, generating an electrical signal proportional to the concentration of the reactant (Knight, 1989). Electrochemical sensors are indifferent to sample colour or turbidity, require little complex equipment and are relatively easily calibrated in aqueous solutions (Buck 1986). They can sense and measure concentrations and activities of ions and neutral species. The functioning of these devices is affected by thermodynamic, kinetic and mass transport factors. For example, response range, response stability (noise drift), response selectivity, response time, lifetime, pretreatment requirements and many others must be considered.

Bienzyme systems that incorporate horseradish peroxidase are frequently used in electrochemical sensors. Substrates that can be monitored voltametrically are used (See Section 1.2.8). The first enzyme in a bienzyme system is usually an oxidase that produces hydrogen peroxide. Fig. 1.5 shows a bienzyme system that utilizes HRP.

$$glucose + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} gluconate + H^+ + H_2O_2$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} H_2O + \text{oxidized chromogen}$$
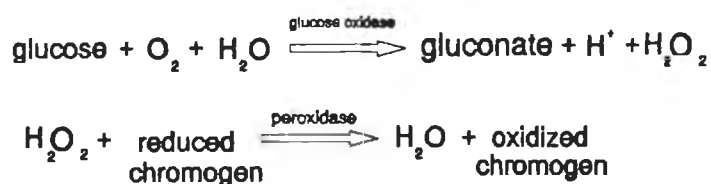
Fig. 1.5.   Bienzyme system using HRP.

A bienzyme sensor for the determination of alcohol, D-amino acid, L-amino acid, choline and cholesterol has been prepared using peroxidase and the respective oxidases (Kulys & Schmid, 1991). The electrodes prepared contained the enzymes immobilized on graphite electrodes that were modified with TTF and TCNQ. The mediator in the system was ferrocyanide. Kulys and Schmid (1990) have also prepared a mediatorless peroxidase bienzyme electrode. The peroxidase used in this

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case was fungal peroxidase from *Arthromyces ramosus*, that does not require a mediator.

Potentiometric devices include pH electrodes, ion-selective electrodes (ISE), gas-sensing electrodes and ion-selective field effect transistors (ISFET). The best example of a potentiometric biosensor is the enzyme electrode which consists of an immobilized enzyme coated over a potentiometric ion-selective or gas-sensing electrode. This device specifically measures substrates or products as they are consumed or produced at the electrode surface (Lowe, 1984). The use of pH electrodes, solid state iodide and cyanide electrodes and gas-sensing electrodes has widened the type of enzyme electrodes available. The electrical potential generated is proportional to the logarithm of the activity of a selected ion in solution (Coughlan et al, 1988). As mentioned previously, there is no consumption of analyte. Ion-selective electrodes follow ion-exchange events occurring at the interface between an electrode covered by a permeability selective membrane and the sample solution. These incorporate ion-selective membranes that allow only the ion of interest, generated in a particular reaction, to permeate and give rise to a signal. Ammonium, fluoride and iodide ion-selective electrodes have been constructed. The main problem with ion-selective electrodes is that they are sensitive to interference from other ions. A number of gas-sensing electrodes have been constructed. Gas-sensing electrodes contain a hydrophobic gas-permeable membrane that separates the sample solution from a pH electrode. Gases such as CO_2 and NH_3, that may be given off in a particular reaction, can permeate the membrane and are detected by a change in pH. Problems associated with gas-sensing electrodes include slow response time and long recovery time. Potentiometric enzyme sensors have also been used in enzyme immunoelectrodes. These combine the specificity and sensitivity of immunoassay procedures together with the usefulness of enzyme electrode technology. Field effect transistors (FET) have been used in ion-selective electrodes to form ion-sensitive field effect transistors (ISFET). An FET is a semiconductor device in which the conductivity is controlled by the strength of the electric field applied to the gate electrode. The gate electrode is part of the integrated circuit that is the electronic component of the biosensor (Coughlan et al, 1988). The semi-

conductor substrate and the metal gate constitute two sides of a parallel-plate capacitor. On application of a voltage between the semiconductor substrate and the gate there is a change in the surface of the substrate layer. Therefore, a conducting channel is formed and a continuous path for the flow of current is generated (Lowe, 1984). CHEMFETs have the gate metal replaced by a chemically sensitive layer. Another type of ion-selective electrode used in conjunction with FETs are metal oxide semiconductor FET (MOSFET), in which the enzymes are immobilized near a palladium-gated metal oxide semi-conductor field effect transistor. MOSFETs have been used to measure gases such as H_2, NH_3 and H_2S. These gases are catalytically decomposed on the oxide electrode, thereby releasing H^+ ions. There is an accumulation of charge at the gate electrode which determines the current flowing in the device, that can be used to detect various gases.

Amperometric biosensors measure the current produced upon application of a constant applied voltage or potential. These sensors measure the flux of an electroactive species that they are designed to detect. They may be used to measure a decrease in the concentration of one reactant, for example, oxygen, or the increase in concentration of a product, for example, hydrogen peroxide (Guilbault & de Olivera-Neto, 1985). In this type of sensor, the analyte undergoes a redox reaction at the sensing electrode and the resultant current is directly proportional to the concentration of analyte present at the surface of the electrode (Coughlan et al, 1988). Because the analyte is consumed in the detection and determination procedure, amperometric measurement is dependent on the rate of mass transfer of the species to the electrode surface. If there is a diffusion barrier to the analyte, such as a membrane, then the rate of mass transfer of the species can be controlled. Most amperometric biosensors are based on the determination of oxygen or hydrogen peroxide. The majority of the employed enzymes operate with the redox system O_2/H_2O_2 as the natural mediator system for the exchange of electrons between the enzyme and the working electrode. An example of an amperometric enzyme electrode is the sensor used to analyze for glucose (Green & Hill, 1986). In this electrode, the disappearance of oxygen, or the appearance of hydrogen peroxide is associated with the glucose oxidase catalyzed reaction, shown in Fig. 1.6.

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconate} + H^+ + H_2O_2$$
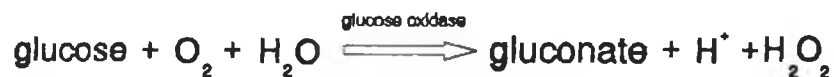
Fig. 1.6.   Glucose oxidase catalyzed reaction.

This reaction can be followed electrochemically, either through the loss of oxygen or by the oxidation of the hydrogen peroxide formed. The Clark type oxygen electrode is used in many amperometric biosensors. This consists of a platinum cathode, with an Ag/AgCl reference electrode, protected by an oxygen permeable membrane. Oxygen is reduced at the cathode giving a current that is proportional to the oxygen concentration in the sample. A wide range of oxidases (including glucose oxidase mentioned above) have been immobilized on oxygen electrodes. The biocatalyst consumes oxygen in the presence of a specific substrate and the signal due to ambient oxygen is reduced in proportion to the concentration of the analyte. Hydrogen peroxide produced by immobilized oxidases can also be monitored amperometrically, using co-immobilized horseradish peroxidase. The oxidase layer can be considered as a means of converting an analyte that is difficult to measure into one that is simple to measure. Hydrogen peroxide can be oxidized at a platinum anode versus an Ag/AgCl electrode. Non-specific oxidation can be eliminated if a cellulose acetate membrane, permeable only to low molecular weight compounds, is placed between the enzyme and the electrode. This is the basis of the first biosensor, commercialized by Yellow Springs Instrument Company, for glucose detection (Hill & Sanghera, 1988).

Problems associated with amperometric sensors include the fact that the large electrode potential may lead to interference from other electroactive compounds. Also, a problem arises from the fact that many of the reactions have an absolute requirement for oxygen as co-substrate. The amperometric determination of peroxide formed by the glucose oxidase reaction at a platinum electrode can be susceptible

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to variations in oxygen tension. An oxygen-independent amperometric sensor with a low operating potential can be constructed to overcome these problems. Oxygen is replaced with an artificial electron acceptor. Alternative electron acceptors to oxygen that allow electrons to shuttle from the enzyme to a suitable electrode material can be used. Synthetic mediators like hexacyanoferrate, chinones or ferrocenes may replace the natural enzyme/electrode system. These acceptors are reduced more rapidly than the competitive reduction of oxygen. Other acceptors include benzoquinone, 2,6-dichlorophenolindophenol sodium, polyviologen, o-chloranil, methylene blue and pyocyanin perchlorate (Lowe, 1984). The acceptors can be electrochemically recycled and can mediate the transfer of electrons between the biological system and the electrode. The reaction for the detection of glucose with a mediator system is shown in Fig. 1.7.

$$\text{glucose} + 2\,(\text{mediator})^{+} \xrightarrow{\text{glucose oxidase}} \text{gluconate} + 2\,(\text{mediator}) + H^{+}$$

Fig. 1.7.   Glucose oxidase catalyzed reaction using a mediator.

The artificial mediator traps electrons produced at the active site of the enzyme and transports them to, or from, the electrode (Green & Hill, 1986). The electrode/mediator system is independent of oxygen and is insensitive to interfering substances (such as uric acid, L-cysteine, reduced glutathione and ascorbate) that are likely to be found in blood and plasma. Various mediators are available, as listed above, but few fulfill all the properties required for incorporation into a biosensor. Ferrocene-modified or tetrathiafulvalene-modified carbon electrodes are usually used in enzyme electrode construction (Coughlan et al, 1988). The mediator is oxidized at the anode, to regenerate the electron acceptor. Oxygen no longer features in the reaction. Use of mediators allows the use of a larger range of oxidoreductases in amperometric sensors. Dimethylferrocene was used as a mediator in an

amperometric biosensor constructed by Cass et al (1984) for the determination of glucose. However, there has been great difficulty in constructing a long-term stable enzyme electrode working with ferrocenes as mediators. Problems arise due to the differences in solubility of reduced and oxidized mediator molecules. Biosensors employing dimethylferrocene or other ferrocene derivatives as mediators, that have low particle size, lose their activity with time because the mediator molecules diffuse away from the electrode surface into the aqueous surroundings with time. Löffler et al (1991) synthesized and studied modified ferrocene derivatives that are completely insoluble in water and are comprised of large molecular weight units. One type of modified ferrocenes studied were monoalkylated ferrocenes with long alkyl chains. These and their oxidized derivatives are insoluble in water. On addition of Tween 20, a surfactant, the insoluble substances form micelles, which are dispersed in aqueous solutions. Thus, an amperometric biosensor with a dispersed mediator system was constructed. A sensitive electrochemical assay for low levels of hydrogen peroxide was described by Frew et al (1986). The system was based on the enzymatic reduction of H_2O_2 by peroxidase and subsequent electron transfer from a gold or pyrole graphite electrode to the enzyme, via a redox mediator.

The third type of electrochemical transducers are conductimetric electrodes. These measure the conductance of the solution to be studied (Lowe, 1984). The conductance in the vicinity of an immobilized enzyme is measured and compared with that of a pair of electrodes lacking immobilized enzymes but placed in the same medium. Electrodes based on conductance are subject to large changes in signal and have not been developed fully into practical biosensors.

1.4.2.2.   Optical and Other Sensors:

Recently, biosensors have incorporated optoelectronic devices and fibre optics. By combining fibre optics and optoelectronic devices with chemical and biochemical reagent systems, it is possible to construct optical biosensors. Photodiodes in conjunction with light-emitting diodes (LEDs) have been used to construct

"optoelectronic" biosensors. In these devices an immobilized biocatalyst or bioreceptor is sandwiched between an LED, or an optical fibre, and the detector (Coughlan et al, 1988). The basic concept of a optoelectronic biosensor is as follows: light from a suitable source is transmitted into the fibre and directed to a region where it can interact with the analyte/sample to be measured, or with a chemical/biochemical transducer. The interaction of light with molecules of the transducer may lead to changes in absorption, transmission, emission, scattering or reflectance of light. This interaction, leading to a change in absorbance or some other parameter, can be collected by the same or another optical fibre and directed to the detector system (Narayanaswamey, 1991). Reactions catalyzed by enzymes can be monitored by using pH sensitive dyes, such as bromocresol green. An optoelectronic device employing bromocresol green can be used to measure serum albumin (Goldfinch & Lowe, 1984). The yellow to blue colour change that is observed when albumin is added to an aqueous solution of bromocresol green is monitored. Polyacrylamide microspheres containing bound phenolsulphonphthalein (phenol red), a pH indicator, can be use in conjunction with smaller polystyrene microspheres that scatter light. Both types of spheres are packed into a cellulose dialysis tubing membrane at the end of a pair of plastic optical fibres. This device is used to measure blood and tissue pH (Lowe, 1984). The device is operated by injecting light from a high intensity tungsten lamp into the illuminating optical fibres. Light is scattered by the polystyrene microspheres and returned via the fibre optic, where it is read at a particular wavelength. Subtraction of one type of light from a reference can be directly related to pH. Use of optical fibre probes allows a high degree of mechanical flexibility, combined with miniature size and low-cost construction. Fibre optics can monitor light emission from a biological element, on its response to illumination. Light is delivered to an immobilized sensing layer at the tip of the fibre optic and absorbance, luminescence or fluorescence can be monitored. At present there is a lot of work being done on optical biochemical sensors (Narayanaswamey, 1991). The advantages of optical biosensors include the fact that non-electrical devices are safer to use when concerned with biological components, samples and applications. Fibre optics are cheap and of high quality. Fibre optic based devices are not subject to electromagnetic interference and do not

require an external reference probe. However, problems that may be encountered with optical fibre biochemical sensors include interference from ambient light and limited ranges. The effect of various factors such as concentration, pH and storage on receptor-based fibre optic biosensors was studied by Rogers et al (1991). An optical immunosensor has been reported by Robinson (1991), in which quantitative measurement of the amount of antibody, antigen or hapten present in a complex sample, such as blood or serum, could be performed.

Other transducers that can be used in biosensors are calorimetric transducers. Nearly all biological reactions are exothermic and so practically all biological reactions can be followed calorimetrically by determining the heat evolved in the catalytic process (Lowe, 1984). The activity of immobilized tissues, microorganisms or enzymes can be monitored in this way (Coughlan et al, 1988). This technique is independent of optical properties of the sample and there are no requirements for auxiliary enzyme reactions normally needed to generate a measurable end product. The enzyme thermistor is a simple device for measuring the heat output when a sample solution is passed through a small immobilized enzyme reactor. The use of immobilized enzyme reactors surrounding the thermistor is favoured over the immobilization of enzymes directly onto the thermistor. Very small temperature differences, of the order of 10^{-2} °C, can be measured. Enzyme thermistors have been used in the areas of clinical chemistry and in fermentation and environmental control (Mosbach, 1991). Specific applications include the detection of enzyme activities, immunological reactions, heavy metal pollutants and the monitoring of enzyme reactor contents, i.e., process control.

Piezocrystal detectors can be used as transducers in biosensors (Coughlan et al, 1988). An alternating field applied across a piezoelectric material, such as quartz crystal, results in small mechanical deformations. Resonance is induced in the crystal at a particular frequency, the frequency being dependent on the crystal mass. The crystal is coated with a layer that selectively absorbs a particular analyte. The change in mass following exposure to the analyte is measured by monitoring the resulting change in frequency. A piezocrystal immunosensor has recently been

developed for the detection of enterobacteria in drinking water, using antibodies against the enterobacterial common antigen (Plomer et al, 1992).

1.4.3.   Immobilization Methods:

The working functions in a biosensor, the biological component and the transducer, must be in intimate contact with each other. In a biosensor the biological component is coupled by immobilization with the physicochemical component, the transducer. It is the transducer that converts the interaction between the analyte and the biological component into a signal. Therefore, immobilization techniques are important in the construction of an operational and accurate biosensor. Immobilization, however, can also function to increase the stability of the immobilized biocomponent. Immobilized enzymes have increased stability, i.e., increased persistence of functional activity, compared with their soluble counterparts (O'Fagain & O'Kennedy, 1991). An immobilization procedure must take into account the fact that the analyte must have access to the biocomponent and also must ensure that no alterations in analyte - biocomponent interactions occur. In some situations it is necessary to restrict access of the analyte to the biocomponent (Coughlan et al, 1988). When using amperometric enzyme electrodes, one must either control the flow of analyte, or restrict its rate of diffusion, so as to avoid dependence on local flow characteristics. The choice of immobilization method largely depends on the nature of the biocomponent to be immobilized. Other factors, such as the type of transducing element, the physical properties of the analyte and the environment in which the sensor is to work must also be considered. The biocomponent must exhibit maximum activity in its immobilized state, must be stable and not prone to leaching. The first immobilized biocomponent must be amenable to the immobilization of a second biocomponent, if necessary. The most common methods used to immobilize a biocomponent include adsorption, covalent attachment, gel/polymer entrapment and crosslinking.

Physical adsorption of the biocomponent on the electrode surface is a popular technique. The procedure for this involves the evaporation of a buffered solution

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containing the enzyme of interest. Adsorption is often followed by crosslinking, as this minimizes leaching of the biocomponent away from the electrode surface (Stoecker & Yacynych, 1990). The advantages of physical adsorption are the fact that no reagents are required and less disruption to the enzyme occurs than with other chemical methods. However, this technique can alter the pH, temperature and ionic optima of the enzyme. Platinum black has been reported to be a good substrate for adsorption, as it has a large surface area and high affinity for proteins.

Crosslinking with bifunctional reagents can be used alone, or in conjunction with physical adsorption and gel/polymer entrapment, to immobilize biocomponents to an electrode surface. Crosslinking can be used to immobilize membranes onto the surface of the electrode. Biocomponents can be entrapped behind these membranes and so immobilized in close proximity to the transducer. Crosslinking that results in the active site of an enzyme not being accessible to the substrate should be avoided. The enzyme would be inactive in this situation and the electrode would not detect any activity. Intermolecular crosslinking between the biocomponent and the electrode surface directly (or indirectly, via a polymer membrane), is favoured. Heterobifunctional reagents, i.e., that have two different functional groups, have been used to immobilize various enzymes onto oxidized silicon (Rusin et al, 1992). The other main immobilization method is that of covalent immobilization. The biocomponent may be covalently linked (this is considered as a form of crosslinking) to a matrix kept in close proximity to the transducer or it may be directly coupled to the surface of the transducing element. Stable immobilized biocomponents are produced in this widely applicable procedure (Stoecker & Yacynych, 1990). It is important that the activity of the biocomponent is not affected. To avoid an adverse effect on activity, covalent immobilization is often carried out in the presence of substrate or competitive inhibitor. The pH and temperature optima of covalently immobilized enzymes may differ from those of native enzyme. Covalent immobilization of enzymes onto support matrices is achieved by reactions including peptide bond formation, alkylation/arylation, diazo linkage and isourea linkage. Peptide linkages are usually directed at the N-terminus of the peptide chain and carboxymethylcellulose and its derivatives are the usual support materials. Protein

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immobilization by diazotisation has also been used to construct biosensors. This method is usually directed at polar side groups. Supports such as starch and cellulose are typically used. Isourea linkage is normally achieved by reaction of isothiocyanate, cyanuric chloride or cyanogen bromide with the lysines or N-termini of proteins. Typical support matrices for such reactions include cellulose- and dextran-based chromatographic materials. Proteins have been immobilized via their N-termini to supports such as carboxymethyl agarose and polyacrylamide. More details of various covalent immobilization reactions can be found in Coughlan et al (1988) and Wilson & Thevenot (1989).

One of the most popular techniques for biocomponent immobilization is gel/polymer entrapment. Many membrane-entrapped enzyme-based biosensors have been reported. In this case, an enzyme solution is contained within the gel/polymer. The enzyme ends up in tiny pores within the gel/polymer. The technique of entrapment and encapsulation includes the retention of biological components behind semi-permeable membranes, i.e., entrapment behind, and microencapsulation within, gels (Coughlan et al, 1988). A wide variety of gels and polymers are available using mild conditions. Entrapment/encapsulation procedures do not involve direct chemical modification of the biocomponents, so that specificity of the interaction between the biocomponent and its analyte is preserved. This immobilization procedure is applicable to enzymes, antibodies and cells. Some commonly used materials for membrane entrapment of biological components in biosensors are cellulose acetate, cellulose nitrate, cellophane, polyvinylalcohol and polyurethane. The membrane properties will influence response time, selectivity and other characteristics. New membrane materials are continuously being produced. Biosensors that incorporate biocomponents by entrapment are only applicable to the detection of small analytes that can readily permeate the membrane. Response time may increase due to a large diffusion barrier (Stoecker & Yacynych, 1990). Commonly used gels include polyacrylamide, alginate, gelatin, agarose and carrageenan. The pore size of the gel must allow maximum access of the analyte while ensuring minimum leaching of the biocomponent.

Immobilization procedures and other factors can bring about reduced operation efficiency and reduced electrode response (Stoecker & Yacynych, 1990). Enhancement of electrode response by a number of methods can be used to overcome these problems. As discussed previously, electron mediators can be used to replace oxygen in oxygen-dependent reactions, so that electrons can be shuttled efficiently between the biocomponent and the electrode. Direct electron transfer means that mediators are not required and here the biocomponent is immobilized directly onto (and not in close proximity to) the transducer (Coughlan et al, 1988). Directly immobilized biocomponents, allowing direct electron transfer, results in faster response times and greater response stability than do indirectly immobilized biocomponents. The reason that direct electron transfer-based biosensors are not more widespread is that biomolecules are not usually compatible with conventional transducing elements. However, ferrocene-modified carbon electrodes and other electrodes made from conducting organic salts, such as TTF TCNQ and NMP TCNQ, are being constructed. Another approach to the enhancement of biosensor response is in the use of multiple enzyme systems (Stoecker & Yacynych, 1990). When the product of a first enzyme can be used as the substrate for the second, enzyme cycling can occur. This enzyme cycling allows a small amount of analyte to create a larger amount of electochemically active product. This creates a greater analytical signal and allows a lower detection limit for the analyte. There are various problems associated with using multiple enzyme systems. It can be difficult to find the best operating conditions for such a biosensor, since the varying requirements of enzyme in the multienzyme system must be taken into account. Also, the sensor performance is limited by the least stable enzyme in the system.

1.5. CONCLUSION:

Horseradish peroxidase can be used in a wide range of applications including analytical, industrial and clinical situations. The stability of this enzyme can be enhanced by a number of chemical modification methods. A wide range of assays are available to monitor HRP activity. All these factors were considered when

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choosing HRP as a target for chemical modification studies. These modifications of HRP will be considered in subsequent Chapters. The attempted production of a stabilized enzyme that could be used in novel situations, such as at elevated temperatures and in other extreme conditions, is discussed.

2.   MATERIALS AND METHODS.

2.1.   Materials:

Peroxidase (E.C.1.11.1.7.) Type VI from horseradish, 3,3',5,5'- tetramethylbenzidine dihydrochloride in powder and tablet form, polyethylene glycol (mol. wt. 15,000 - 20,000), borax, N α-acetyl-L-lysine, picryl sulphonic acid (trinitrobenzenesulphonate), ethylenediaminetetraacetic acid (EDTA), albumin (bovine), sodium dodecyl sulphate, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium sulphate, tris[hydroxymethyl]aminomethane, glycerol, L-cysteine, Coomassie brilliant blue (G-250 for Bradford protein assay and R-250 for SDS-PAGE protein stain), triethanolamine HCl, N,N-dimethyl formamide, sodium hydroxide, guanidine hydrochloride, 5,5'dithio-bis-(2-nitrobenzoic acid) (DTNB), 2-[N-morpholino]ethane sulphonic acid (MES) and all crosslinking and chemical modification reagents were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

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

Sephadex G-25 and DEAE-Sepharose were obtained from Pharmacia, Uppsala, Sweden.

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

All other reagents were of analytical grade from Merck, Germany, BDH Ltd., Poole, England, or from Riedel de Haen, Germany.

Enzfitter was obtained from Biosoft, Cambridge, U.K.

2.2. Optimization of Horseradish Peroxidase Microassay:

A colorimetric enzyme microassay for horseradish peroxidase (HRP) was developed based on the method of Bos et al (1981) and Gerber et al (1980). Horseradish peroxidase was prepared at a concentration of 1 mg/ml in 0.1M phosphate buffer pH 7.0. Serial dilutions from the enzyme stock were made in 10mM phosphate pH 7.0 + 0.002% Tween 20 to a concentration of 0.1µg/L HRP. The hydrogen donor tetramethylbenzidine dihydrochloride (TMB) was used in powder or tablet form at a concentration of 0.1g/L. Dimethyl sulfoxide (at 2% final volume) was used to initially dissolve powder TMB. One TMB tablet containing 1mg of TMB was dissolved in 10ml buffer as outlined by the manufacturers. The buffer used was 0.1M sodium citrate pH 4.0. Just before assay 30% v/v hydrogen peroxide was added to TMB solution at a concentration of 0.03%, i.e., 3µl hydrogen peroxide per 10ml TMB.

50µl/well of each HRP dilution was pipetted in quadruplicate into a 96 well microtitre plate. The plate was equilibrated at 25°C in a Titertek Twinreader Plus microplate reader from ICN-Flow Laboratories. 150µl of buffered substrate solution (i.e. TMB + H_2O_2) was dispensed into each well to start the reaction using the dispensing option on the platereader. The mixture in the wells turned blue as reaction proceeded. Blank wells contained 50µl 10mM phosphate buffer Ph 7.0, in place of HRP. The platereader was programmed to shake the plate directly after addition of buffered substrate solution. Absorbance values at 620nm and 690nm were read at various intervals using kinetic programme on platereader. Alternatively, the reaction was terminated after a certain interval with 50µl 2M sulphuric acid per well. After addition of the acid wells turned yellow and the absorbance at 450nm was read. Each activity determination was the mean of n wells, i.e., replicate wells were used in all cases. Standard assay conditions were determined as: A_{620}nm read after 2 minutes at enzyme concentration of 66.7µg/L.

2.3.   Purification of Horseradish Peroxidase:

Horseradish peroxidase was purified by ion exchange chromatography based on the method of Tijssen & Kurstak (1984). DEAE-Sepharose was equilibrated in 250mM phosphate buffer pH 8.0 and packed into a 10.0 cm³ column. 2.5mM phosphate buffer pH 8.0 was passed through the column. 1ml of 1mg/ml horseradish peroxidase in 2.5mM phosphate pH 8.0 was applied to the column. 80 x 500μl fractions were collected and assayed for enzyme activity using standard assay. Fractions containing enzyme activity were concentrated by dialysis against polyethylene glycol. Protein concentration of resulting isoenzyme C (see Tijssen & Kurstak 1984) was determined by bicinchoninic acid assay (Smith et al, 1985) using bovine serum albumin as standard. Dilutions of isoenzyme C were prepared in 10mM phosphate pH 7.0 + 0.002% Tween 20 and optimum assay conditions were determined as was done for horseradish peroxidase standard assay. Stock horseradish peroxidase and isoenzyme C were analyzed by SDS-polyacrylamide gel electrophoresis as described in Section 2.4.

2.4.   SDS-Polyacrylamide Gel Electrophoresis:

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). A 10% resolving gel and a 3% stacking gel were used. Table 2.1 gives details on the preparation of gels. Sodium dodecyl sulphate is a detergent that will unfold proteins and was included in gels at a concentration of 10% w/v. Non-reducing conditions (no mercaptoethanol) were used in gels. The "acryl/bis-acryl" was prepared by dissolving 30g acrylamide and 0.8g bis-acrylamide in 100ml H_2O. The running buffer had a pH of 8.0 and contained Tris (0.025M), glycine (0.192M) and SDS (10% w/v). The solubilization buffer for sample solubilization was prepared as follows: 0.969g Tris + 10ml glycerol + 0.05ml bromophenol blue made up to 100ml with H_2O. Samples for electrophoresis were boiled in an equal volume of solubilization buffer for 5 minutes. 10μl molecular weight markers and 20 - 40μl sample volumes were used and samples were applied to gel under running buffer.

Molecular weight markers in the range of 29,000 - 205,000 daltons were used.

The resolving gel was prepared as described in Table 2.1 and poured to within 2 - 3 cm of top of plate and overlaid with water. After 20 - 30 minutes, by which time the gel had set, the water was poured off and the stacking gel was poured. The comb was then inserted for 10 minutes. Gel plates etc., were assembled in electrophoresis apparatus and covered in running buffer. Samples were then applied. A constant current of 25mA per gel was used with a running time of 3 hours (or until dye runs off end of gel). Electrophoresis equipment used was an Atto minigel system with corresponding power pack.

Table 2.1.   Details of preparation of gels for SDS-polyacrylamide gel electrophoresis.

TYPE OF GEL	RESOLVING (10%)	STACKING (3%)
STOCK SOLUTION	VOLUME (ml)	VOLUME (ml)
Water	14.1	9.3
"Acryl/bisacryl"	10.5	1.25
1.87M Tris HCl pH8.8	6.3	—
0.5M Tris HCl pH6.8	—	0.60
SDS (10% w/v)	0.15	0.05
TEMED[*]	0.03	0.05
Ammonium per-sulphate (10% w/v)	0.15	0.04

[*] TEMED acts as a catalyst for gel polymerization so it is added last.

Gels were placed in stain for approx. 15 - 20 minutes and then placed in destain. Stain was prepared with 0.5% w/v Coomassie brilliant blue in acetic acid : methanol: water at a ratio of 1 : 8 : 10. Destain is prepared in the same way but without the inclusion of Coomassie brilliant blue.

2.5. Bis-imidate Modification:

Three bis-imidates were used: dimethyl suberimidate, dimethyl pimelimidate and dimethyl adipimidate. Methods used were based on de Renobales and Welch (1980), Minotani et al (1979), on Sheehan et al (1990) and on O'Fagain et al (1991(a)).

2.5.1. Crosslinking with Dimethyl Suberimidate:

Horseradish peroxidase (1mg/ml) was prepared in 0.1M phosphate buffer pH 7.0 or pH 8.0. The isoenzyme purified in Section 2.3 was used at concentration and pH obtained after purification, i.e. 20 - 60 mg/L, pH 7.0. Dimethyl suberimidate was used at final concentrations ranging from 0.25µg/ml to 5.0mg/ml in 0.1M phosphate pH 8.0. The solutions prepared were added to enzyme at a final volume of 2% followed by vigorous mixing. Solutions of dimethyl suberimidate were used immediately after preparation as bis-imidates have a short half-life in solution. The crosslinking reaction was allowed to proceed at room temperature for 1 hour.

After 1 hour samples were diluted with 10mM phosphate pH 7.0 + 0.002% Tween 20 and assayed for enzymatic activity using standard conditions to determine initial recoveries.

Thermoinactivation of native and modified samples was studied by incubating native and modified samples (66.7µg/L) at approx. 72.5°C for 60 minutes. Aliquots of each sample were withdrawn onto ice at various intervals over the 60 minutes and assayed for enzyme activity using standard conditions. % Relative activity was then determined, i.e., enzyme activity remaining at time aliquot was taken relative to

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enzyme activity that was present at start of incubation. % Relative catalytic activity was plotted vs. time to see pattern of thermoinactivation of the samples.

Titration of amino groups on control and modified samples was performed using the trinitobenzenesulphonate (TNBS) assay, as described in Section 2.6.

Native and modified stocks were treated with sodium borohydride by the method of Tijssen (1985), as described in Section 2.7.1.

2.5.2.   Crosslinking with Dimethyl Pimelimidate:

Method used to crosslink HRP with dimethyl pimelimidate was the same as for dimethyl suberimidate modification experiments. (See Section 2.5.1). Dimethyl pimelimidate was used at concentrations ranging from 0.1 to 10.0mg/ml.

The determination of initial recoveries and % relative catalytic activity and titration of amino groups were performed as for dimethyl suberimidate. Borohydride treatment of native and modified stocks was based on the method of Blanco & Guisan (1989). (See Section 2.7.2).

2.5.3.   Crosslinking with Dimethyl Adipimidate:

Procedure to crosslink HRP with dimethyl adipimidate was as described for dimethyl suberimidate in section 2.5.1. Dimethyl adipimidate was used at concentrations ranging from 0.1 to 10.0 mg/ml.

Initial activity check and % relative catalytic activity were determined as described in Section 2.5.1. SDS-polyacrylamide gel electrophoresis of native and modified stocks was carried out as described in Section 2.4. Borohydride treatment of DMA samples was performed according to the method of Tijssen (1985) as described in

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Section 2.7.1. High performance liquid chromatography (HPLC) was also carried out on samples. See section 2.10. for details.

2.6.   Determination of Amino Groups:

Amino group determination was performed using trinitrobenzenesulphonate (TNBS) according to Fields (1971). A sample of protein (centrifuged or uncentrifuged - See section 2.8.) was added to 0.5ml borate buffer (0.1M $Na_2B_4O_7$ in 0.1M NaOH, pH 9.5) and volume was made up to 1ml with H_2O. 20µl of a 1.8M TNBS solution was added to sample which was then mixed. Reaction proceeded for 5 minutes and was stopped with 2ml 0.1M NaH_2PO_4/1.5mM sodium sulphite solution. Absorbance at 420nm was determined against blank (i.e. no protein). Readings were taken using a Shimadzu recording spectrophotometer. Standards of N α-acetyl-L-lysine (i.e. 1 free amino function) in concentration range of 0.1 - 10mM were used.

2.7.   Borohydride Treatment of Horseradish Peroxidase:

Native and modified enzyme samples were treated with sodium borohydride in an attempt to reduce any Schiff bases present in samples. Two methods were used:

2.7.1   Borohydride Treatment Based on Method of Tijssen (1985):

1mg/ml sodium borohydride in 0.1mM NaOH was added to native or modified enzyme sample at 5% final volume. Sample was mixed and 30 minutes later fresh 1mg/ml sodium borohydride in 1mM NaOH was added at 10% final volume. Sample was then incubated at 4°C for a minimum of 1 hour. Initial recoveries and % relative catalytic activity were determined as before.

2.7.2. Borohydride Treatment Based on Method of Blanco & Guisan (1989):

50mM sodium borohydride in 50mM borate buffer pH 10.0 was prepared and added to an equal volume of native or modified enzyme sample. (Final borohydride concentration = 25mM). Sample was left at room temperature overnight. Initial recoveries and % relative catalytic activity were determined as before.

2.8. Micro-column Centrifugation:

This procedure was based on the method of Helmerhorst & Stokes (1980). Sephadex G-25 was swollen in buffer of choice and packed into 1.0, 2.5 or 5.0ml column. The column was centrifuged at 1,400 x g for 2 minutes to remove excess buffer. Sample was then applied at approx. 5 - 10% column volume and column was re-centrifuged at 1,400 X g for 2 minutes. The eluate from each column was collected.

2.9. Thermoinactivation of Horseradish Peroxidase:

Enzyme samples at a concentration of 66.7μg/l (i.e. standard assay concentration) in 10mM phosphate pH 7.0 + 0.002% Tween 20 were incubated at various temperatures from 45°C to 75°C. Aliquots of each sample were withdrawn onto ice over a 60 minute period and each was assayed for % relative catalytic activity as described in Section 2.5.1. % Relative catalytic activity vs. time for each temperature was plotted and kinetics of thermal deactivation were analyzed as per Section 2.10.

2.10. Kinetics of Thermoinactivation of Horseradish Peroxidase:

First and second order decay curves were plotted and corresponding rate constants were determined for results obtained in Section 2.9 using the Enzfitter programme.

Results from the temperature at which decay over time was uniform were used.

2.11. High Performance Liquid Chromatography:

A Beckman System Gold was the HPLC apparatus used. A Protein Pak SW 300 was used to separate native and modified samples. This column allows larger sized molecules to elute first. Mobile phase used was 0.1M phosphate buffer pH 7.2 which had been filtered and degassed. Programme used had a running time of 30 minutes and measured absorbance at 280nm. Peaks appeared in areas where protein was present and retention times of these peaks were given. Peak areas and retention times were studied in order to distinguish any differences in molecular size of applied samples.

2.12. Methylacetimidate Modification of Horseradish Peroxidase:

Method used to modify HRP with methylacetimidate was as for bis-imidates as described in Section 2.5. Target protein was 1mg/ml horseradish peroxidase pH 7.0 or isoenzyme C. Methylacetimidate was added in phosphate buffer pH 8.0 at 2% final volume or as a dry powder at concentrations ranging from 0.01mg/ml to 5.0mg/ml. Modification reaction proceeded at room temperature for 2 hours. Initial recoveries and % relative catalytic activity were determined as before (See Section 2.5.1). Borohydride treatment of native and modified stocks was according to Section 2.7.1.

2.13. Activation of Carboxyl Groups on Horseradish Peroxidase:

1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), a water soluble carbodiimide, was used to activate carboxyl groups on HRP. EDC was prepared at concentrations ranging from 1mM to 1M in either 0.1M MES buffer pH 5.0 or 0.1M

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phosphate buffer, pH 7.0. EDC solutions were added to 1mg/ml horseradish peroxidase pH 7.0 at 10% final volume. Solutions were mixed and reaction was for 1 hour at room temperature after which crosslinking with a diamine was carried out as per section 2.14.

2.14. Crosslinking with a Diamine:

Diamine used to crosslink activated HRP was 1,2-diaminoethane. The concentration of 1,2-diaminoethane used corresponded to concentration of carbodiimide used, i.e., 10mM carbodiimide treated sample was mixed with 10mm 1,2-diaminoethane, etc. Crosslinking reaction was for 1 hour at room temperature. Initial recoveries and % relative catalytic activity were measured as before (See section 2.5.1).

2.15. Crosslinking with N-hydroxysuccinimide Bis-Esters:

Esters used were suberic acid bis-ester of N-hydroxysuccinimide and ethylene glycol bis-succinic acid ester of N-hydroxysuccinimide. N-hydroxysuccinimide (NHS) was used as a monofunctional control.

2.15.1. N-hydroxysuccinimide Modification Experiment:

Procedure for N-hydroxysuccinimide modification of HRP was based on the method of Ji (1983), Partis et al (1983) and Sheehan et al (1990). To 1ml of 1mg/ml horseradish peroxidase pH 8.0 was added 1mg of N-hydroxysuccinimide ester dissolved in 5%(v/v) DMSO. Modification reaction was at room temperature for 20 -30 minutes and was terminated by the addition of an equal volume (i.e. 1050µl) of cold 0.1M Tris HCl pH 7.0. Samples were diluted to standard assay concentration and assayed for initial recoveries and % relative catalytic activity as before (See Section 2.5.1).

2.15.2. HPLC on N-hydroxysuccinimide Modified Samples:

Native and NHS modified samples were analyzed by HPLC as per Section 2.10.

2.15.3. Determination of U.V./Visible Spectra of NHS Modified Samples:

Spectra of native and modified NHS samples (at 0.5mg/ml, i.e. concentration after modification reaction) were determined using the spectrum mode on the Shimadzu recording spectrophotometer. Absorbances were read over a wavelength range of 280nm to 540nm.

2.15.4. Construction of pH Profile:

10mM phosphate buffer was prepared at various pH values ranging from 4.5 to 8.5. Each of these buffers was then used to dilute native and NHS modified samples and enzyme activity was determined using standard assay conditions (except for variation in pH value of diluent). A plot of enzyme activity vs. pH was constructed.

2.15.5. Fluorescent Emission Spectra:

Fluorescence emission spectra of native and NHS-modified samples at various concentrations were studied with a Perkin Elmer LS50 luminescence spectrophotometer. Scans of fluorescent emission were recorded from 200nm to 700nm. An excitation wavelength of 280nm and slit width of 2.5nm were used. (See Schmid, 1989).

2.15.6. SDS-Polyacrylamide Gel Electrophoresis:

Native and NHS modified samples were analyzed by SDS-polyacrylamide gel electrophoresis according to Section 2.4.

2.15.7. Stability towards Inactivation by Guanidine HCl:

6M guanidine HCl (GnCl), a protein denaturant, was prepared by dissolving 1.009g GnCl in 1.0g H_2O (See Pace 1989). An equal volume of GnCl was added to native or modified sample to give final GnCl concentration of 3M. Reaction proceeded at room temperature for 3 hours after which 600µl 0.1M phosphate was added to samples. Samples were diluted to standard assay concentration and assayed for enzyme activity using standard conditions to determine the effect of the denaturant on native and modified stocks.

Following overnight incubation of GnCl treated stocks, 25mM 2-mercaptoethanol, a reducing agent, was added to GnCl-treated native and modified samples. These samples were stored overnight, diluted to standard assay concentration and assayed for enzyme activity to assess the effect of denaturant combined with a reducing agent.

2.16. Modification of Horseradish Peroxidase with 2-Iminothiolane:

Procedure for modification of HRP with 2-iminothiolane in an attempt to introduce sulfhydryl groups into the enzyme molecule is based on the method of Kenny (1979). 500mM 2-iminothiolane was prepared in 0.1M phosphate and 1mM EDTA, pH 8.0. Stock horseradish peroxidase of 1mg/ml was prepared in same buffer. 24µl of 2-iminothiolane stock was added to 1ml enzyme to give final iminothiolane concentration of 12mM. Solutions were mixed and incubated at 0°C for 2.5 hours. After reaction, a volume of sample was centrifuged in 0.1M phosphate pH 7.3 and

1mM EDTA as per Section 2.8. Centrifuged sample and uncentrifuged sample were assayed by Ellman's assay for thiol group determination as in Section 2.18. Remainder of 2-iminothiolane treated samples were modified with heterobifunctional reagents. (See section 2.17).

2.17.   Modification using Heterobifunctional Reagents:

Samples from Section 2.16 were treated with γ-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS) and 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (4NM) based on the method of Yoshitake et al (1982). 2-Iminothiolane samples were subjected to micro-column centrifugation in 0.1M phosphate and 1mM EDTA pH 6.0. To resulting fractions was added 0.01g GMBS dissolved in 20µl DMF or 0.01g 4NM dissolved in 20µl DMF. Samples were mixed and stored at 4°C overnight to allow for reaction of heterobifunctional reagents with thiol groups on enzyme protein.

After overnight incubation, samples were assayed for number of thiol groups as per Section 2.18 and subsequently centrifuged in phosphate buffer pH 7.0 using micro-column centrifugation. Eluted fractions were incubated at 30°C for 1 hour to allow for reaction of amino groups with the heterobifunctional molecule. Fractions were then assayed for thiol group content as per Section 2.18. Initial recoveries and % relative catalytic activity were determined as before.

2.18.   Ellman's Assay for Thiol Group Determination:

This was performed as described by Creighton (1989). Protein samples were prepared in 0.1M phosphate buffer and 1mM EDTA, pH 7.3. All assay reagents were incubated at 25° C in thermostatted compartment of Shimadzu spectrophotometer and assay was performed at 25° C. Absorbance at 412nm of protein sample (1.5ml) was measured relative to blank (1.5ml). 50µl of 3mM 5,5'-

dithio(2-nitrobenzoic acid) (DTNB) in 0.1M phosphate pH 7.3 was added to each cuvette and mixed. Increase in absorbance at 412nm was followed and when absorbance reached a steady value, a reading was taken. The molar concentrations of thiols was calculated using the molar absorbance of TNB anion [ε_{412} = 1415/M cm] (Creighton, 1989).

3.　　STUDIES ON NATIVE HORSERADISH PEROXIDASE

3.1.　　Horseradish Peroxidase Microassay Optimization:

3.1.1.　　Introduction:

Many hydrogen donors form coloured products on oxidation and this is particularly useful in the development of a colorimetric assay. Commonly used hydrogen donors that form coloured products on oxidation, referred to as chromogenic substrates, are: o-phenylene diamine (OPD), 2,2'-azino-di-(3-ethyl-benzthiazoline-sulphonate) (ABTS) and the one chosen for use in this case; 3,3',5,5'-tetramethylbenzidine (TMB). HRP catalyzes the H_2O_2 oxidation of these substrates by transferring electrons from the hydrogen donor to the peroxide to yield a coloured product. TMB, which has an absorption spectrum showing three peaks at 370nm, 655nm and 450nm, is superior to some of the other hydrogen donors as it is extremely sensitive and is non-carcinogenic and non-mutagenic (Liem et al, 1979, Bos et al, 1981 and Holland et al, 1974). TMB has a rapid reaction rate and is ideal for use in kinetic enzyme assays and in immunoassays. In end point assays TMB is extremely sensitive and allows for quantitative measurements. Due to its high sensitivity, TMB is more economical to use than other substrates in terms of quantity of enzyme and antibody samples that are needed. The assay for TMB was optimized in an attempt to develop a microplate assay that could be used as a standard procedure.

3.1.2.　　Results and Discussion:

3.1.2.1.　　TMB Assay Optimization:

A microassay for horseradish peroxidase was developed utilizing the hydrogen donor 3,3',5,5'-tetramethylbenzidine (TMB) as described in Section 2.2. The HRP-TMB microassay was optimized with respect to pH, enzyme concentration, time, wavelength and temperature. The pH range of HRP is in the range of 4.0 - 8.0 (Paul, 1963). Preparation of a 1 mg/ml stock solution of HRP in 0.1M phosphate

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buffer pH 7.0, that can be stored at 4°C, was based on information obtained from various suppliers of HRP and from Tijssen (1985). HRP may be inactivated by polystyrene microtitre plates if Tween 20 is omitted (Berkowitz & Webert, 1981) so Tween 20 is added to diluent (10mM phosphate buffer pH 7.0) at 0.002% v/v. Horseradish peroxidase activity can be measured indirectly by following the rate of transformation of the hydrogen donor. A change in colour of the reaction mixture in the wells of microtitre plate from colourless to blue was taken as an indication of HRP activity. The intensity of the blue colour is proportional to the concentration of enzyme present in the well. Absorbance values were directly correlated to enzyme activity. A linear increase in absorbance with time was desired.

50µl enzyme sample and 150µl TMB buffered substrate solution were the assay volumes. These were similar to volumes used by Bos et al (1981).

The assay was carried out at 25°C. This temperature was as used previously by Bos et al (1981) and Gerber et al (1985). Also, the traditional HRP assay involving guaiacol as hydrogen donor was carried out at 25°C (See Paul, 1963). Plates, containing enzyme samples only, were incubated for approx. 5 minutes at this temperature in the Titertek Twinreader Plus platereader (which has a temperature control function).

A range of horseradish peroxidase concentrations from 0.1 µg/L to 100.0 µg/L were assayed at various intervals at a wavelength of 620nm or 690nm. Although TMB has an absorption peak at 650nm, no filter for this wavelength was available. It was decided to use the 620nm filter for the assay. See Fig.3.1 (a, b and c) for graphs of absorbance at 620nm vs. time for some of the HRP concentrations studied. Recently an assay for TMB that reads away from the absorption maximum, using dual wavelength readings, has been described (Madersbacher & Berger, 1991). The use of one wavelength readings is more convenient. As mentioned in Section 2.2, the HRP-TMB reaction can be terminated by addition of a strong, non-oxidizing acid such as 2M H_2SO_4 (Bos et al, 1981). The colour of the substrate reaction product turns from blue to bright yellow due to loss of two electrons under acidic conditions.
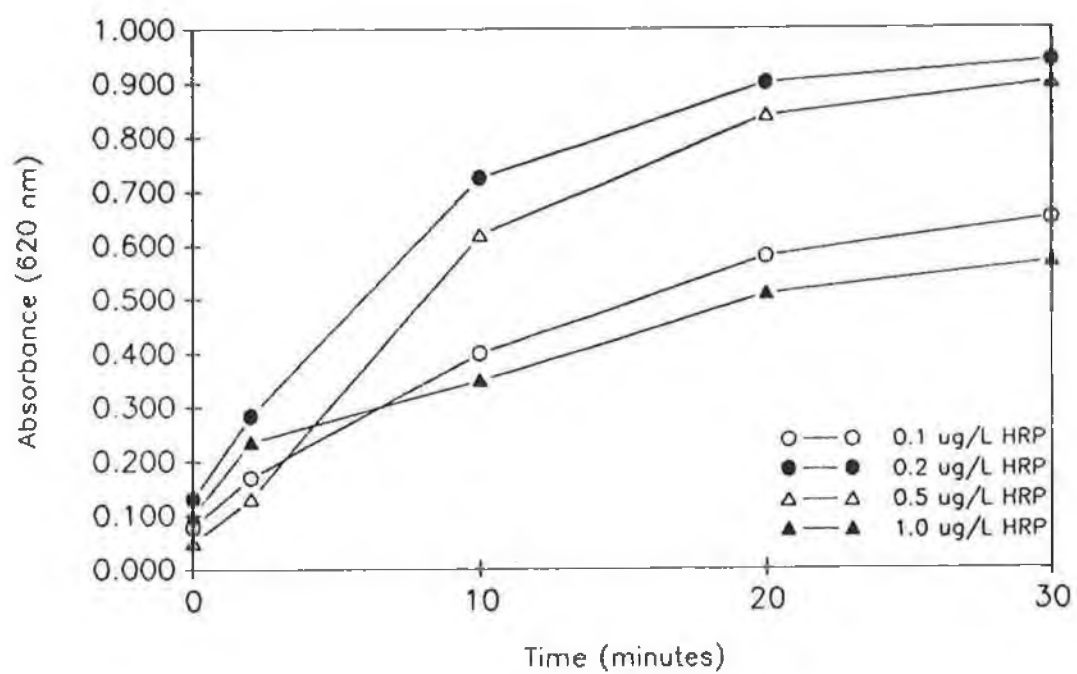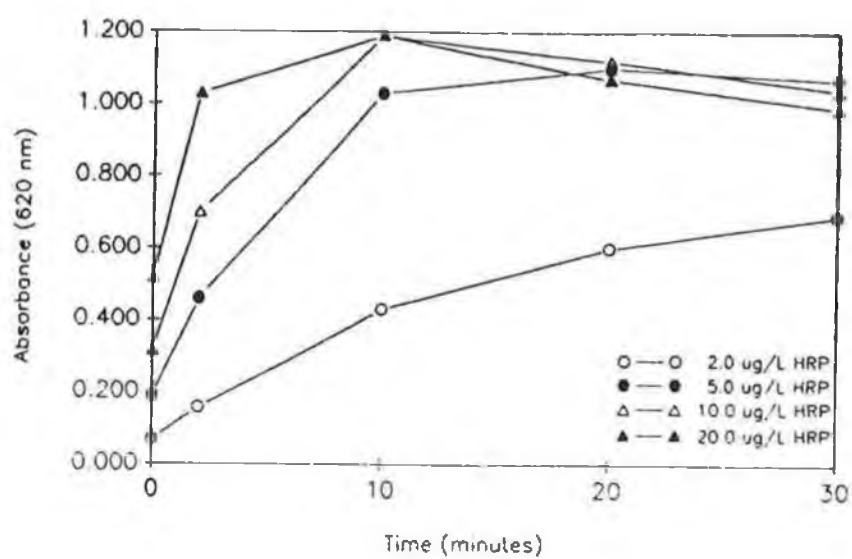
Fig. 3.1 (a)   Absorbance at 620nm vs. time for various HRP concentrations.
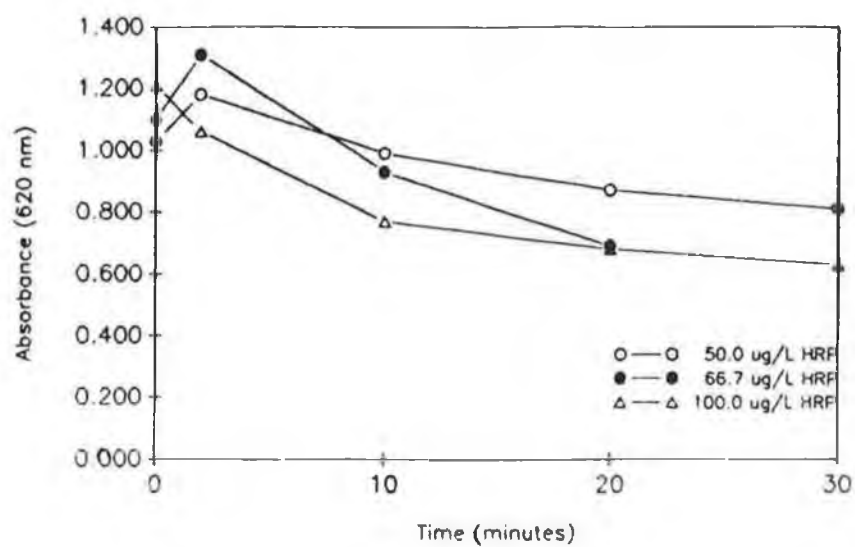
(b)

(c)

Fig. 3.1 (b) and (c)   Absorbance at 620nm vs. time for various HRP concentrations.

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The absorption maximum of the reaction mixture shifts to 450nm. This peak at 450nm is only very small prior to addition of the acid. The use of acid in the TMB assay was not pursued as it required an extra step and involved the use of a hazardous substance. The use of acid is said to increase the sensitivity of the assay 2 - 4 fold. However, adequate readings were obtained at 620nm. The optimum assay conditions were determined as: 66.7µg/L HRP read after 2 minutes at 620nm at approx. 25°C. These conditions give an absorbance value of over 1.2 (See Fig.3.1(c)). This was the highest value seen and that is why these conditions were chosen as optimum, standard assay conditions. Using a concentration of 66.7µg/L, the reaction showed a linear increase in absorbance over time for 2 minutes only (See Fig.3.1(c)). It appears in some of the graphs as that the starting absorbance is quite high. The assay was started with colourless wells in these cases. However, the reaction is extremely rapid and proceeds faster than the platereader is able to measure. Assays for HRP using other hydrogen donors have been reported where reaction is linear for only 2 minutes (Shindler et al, 1976) so this time period should be sufficient. Gerber et al (1985) state that it is advisable to utilize the initial reaction period during which enzyme activity is most linearly related to enzyme concentration. The assay results were reproducible and uniform when assayed under the standard assay conditions mentioned above. A high absorbance value was desired in the standard assay. This standard assay is to be used to determine differences between native and modified peroxidase forms in denaturation curves. A standard assay with a high absorbance value will be able to observe easily any fine or gross differences in stability between different enzyme forms. A time period of 2 minutes is used as it is a convenient time period in which to measure a number of samples.

Tetramethylbenzidine dihydrochloride powder was finally chosen as the form of TMB to be used. Initially, TMB solutions from two suppliers, i.e., Noctech and Serex were used. Results from use of powder and tablet forms of TMB purchased from Sigma Chemical Co. gave higher overall absorbance readings than the other products and appeared more sensitive at lower enzyme concentrations. Because of this, and due to the fact that the powder and tablet forms of TMB were commercially

available from a recognized and reliable source, optimization of the assay for Sigma TMB only was undertaken. TMB in the dihydrochloride form is described as being water/buffer soluble. However, problems were encountered in dissolving the dihydrochloride form of TMB, even at low concentrations. Bos et al (1981) use various organic solvents to dissolve TMB free base, which is not water soluble, as do Gerber et al (1985). The manufacturers recommend the use of dimethyl sulfoxide (DMSO) to aid dissolution of TMB free base only, not the dihydrochloride form. It was found necessary to use DMSO (at 2% buffer volume) to initially dissolve the powder form of TMB dihydrochloride before dissolving further in buffer. Although the TMB tablets were used according to the manufacturer's instructions, they did not fully dissolve in the recommended 0.05M phosphate-citrate buffer. Also, the tablets were not as economical to use as the powder. So, only the powder was used in further assay optimization. Various sodium and potassium phosphate - citrate buffers of pH 4.0 - 5.5 were used and also sodium acetate buffer pH 6.0 to dissolve powder TMB dihydrochloride. The pH range for TMB should be in the region of 4.0 - 8.0 (Gerber et al, 1985). Therefore, TMB should dissolve in buffers with a pH in this range. Concentrations of TMB in the range of 1mg/100ml to 10mg/100ml were prepared. Any concentration higher than this would not dissolve. A concentration of 10mg/100ml or 0.1g/l TMB in 0.1M sodium - citrate buffer pH 4.0 gave highest optical density readings and appeared clear in solution. Therefore, this was used in all subsequent assays.

The optimum concentration of hydrogen peroxide was found to be 0.03%. Concentrations of hydrogen peroxide higher than this gave low optical density readings. This occurs because HRP has a critical concentration for hydrogen peroxide (Gerber et al, 1985 and Tijssen, 1985).

Parameters determined for HRP-TMB standard assay are in accordance with details found in the literature on TMB used in enzyme assays, immunoassays and immunohistochemistry, especially those details given by Bos et al (1981). TMB is a very sensitive and useful hydrogen donor due to the intense colour it produces on oxidation and due to its non-carcinogenic activity. The only problem encountered

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in the use of TMB as a hydrogen donor was in the care that had to be taken to avoid TMB oxidation prior to use. All glassware used with TMB must be very clean, TMB solution must be stored at 4°C in a sealed, covered container after preparation and should only be made up on day of use. Also, hydrogen peroxide should be added immediately before assay and the buffered substrate solution used within a few minutes. If the above precautions are not taken high background colour in blank wells may result or solution may oxidize, i.e., turn blue before use (See Liem et al, 1979).

3.1.3. <u>Summary</u>:

A microassay for horseradish peroxidase using the highly sensitive, non-mutagenic and non-carcinogenic hydrogen donor 3,3',5,5'-tetramethylbenzidine dihydrochloride was developed. The standard conditions used in the assay are similar to and agree with those cited by Bos et al (1981). The standard assay parameters are: 66.7µg/l HRP concentration, 0.1g/L TMB in 0.1M sodium - citrate pH 4.0 containing 2% v/v DMSO, 0.03% hydrogen peroxide concentration, 2 minute incubation time and absorbance reading at 620nm. Using these parameters a linear increase in absorbance over time is seen for 2 minutes and a high absorbance reading is seen at this time. This assay is used as the standard assay in chemical modification work (See Chapter 4). The advantages of the assay are the high sensitivity and hence the low enzyme concentration needed, absence of toxic effects from reagents and short incubation and assay time. When a microplate reader with automatic dispensing and incubation functions is used experimental error is decreased and a large number of samples can be read in a relatively short time. The assay is also reproducible so it can be used as a standard control when studying effects of chemical modification experiments and other situations.

3.2 Purification of Horseradish Peroxidase:

3.2.1. Introduction:

Horseradish peroxidase type VI from Sigma Chemical Co. is a relatively pure form of peroxidase. However, this and other commercially available peroxidases are reported to contain several isoenzymes (Tijssen, 1985 and Tijssen & Kurstak, 1984). In total seven isoenzymes of horseradish peroxidase have been isolated and purified to homogeneity (Shannon et al, 1966). The C isoenzyme dominates to a large extent and has the highest activity (Tijssen, 1985 and Welinder, 1979). The other isoenzymes present have much lower activities. The purification of horseradish peroxidase by DEAE-Sepharose chromatography was performed by Tijssen & Kurstak (1984) to remove inactive proteins and low activity isoenzymes from commercially pure peroxidase. Tijssen & Kurstak were using the purified peroxidase for the preparation of peroxidase - antibody conjugates. Therefore it was necessary to remove all inactive protein that may bind to antibody and lead to low yield of active conjugates. Similarly, for application of peroxidase in chemical modification procedures it was necessary to ensure that a highly active and pure peroxidase was being modified. Any step that would increase the purity of the enzyme and hence the efficiency of the modification procedure would be advantageous to carry out. In this way chemical modifiers would bind to highly active peroxidase and not to inactive apoenzyme or contaminating protein found in commercial preparations of peroxidase.

3.2.2. Results and Discussion:

3.2.2.1. Horseradish Peroxidase Purification by Ion Exchange Chromatography:

Horseradish peroxidase was purified by ion exchange chromatography as described in Section 2.3. This procedure was based on the method of Tijssen & Kurstak (1984). In this procedure the HRP did not adsorb to the column and was eluted without any change in ionic strength. A typical elution profile is shown in Fig. 3.2.

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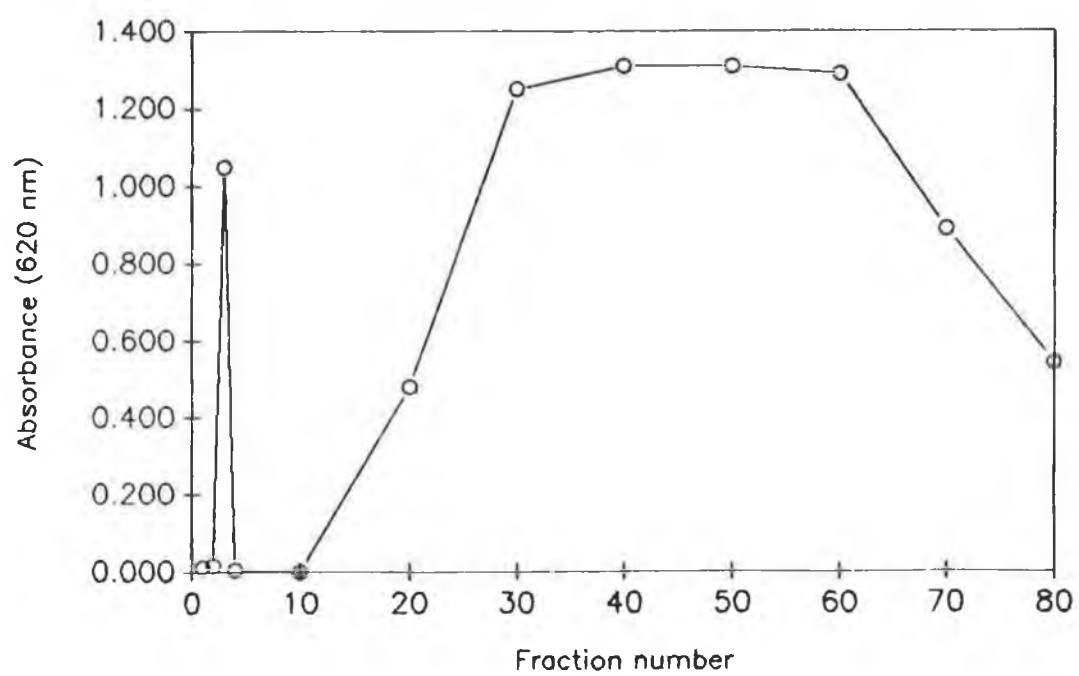
Fig. 3.2.   Elution   profile   of   HRP   fractionated   by   ion   exchange
chromatography.

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As can be seen, two peaks emerged; a sharp, narrow one and a broad, large one. For the broader peak, the most active fraction obtained represents 100% recovery of activity of applied sample, i.e., the most active eluted fractions have the same activity as the applied sample. For the narrow peak, the most active fraction represents 80% of the activity of applied sample, i.e., there is 80% recovery of activity of applied samples in the most active fractions. The activity recovered in the narrower peak is present in one fraction only. For all fractions, even high activity ones, there is very low protein content. The protein content of eluted fractions was determined by the Bradford, ultraviolet (A_{280}) and bicinchoninic acid protein determination methods. In all methods protein content was virtually indeterminable.

Tijssen & Kurstak applied an ionic strength gradient to elute three components; a highly active component, presumably isoenzyme C, a less active isoenzyme in the second component and low peroxidase activity and contaminants in the rest of the fractions, or the third component. Therefore, looking at Fig. 3.2 it is likely that fractions 30 - 60 constitute isoenzyme C and that the peak at fraction 3 is a less active, less abundant, isoenzyme. Other inactive proteins and contaminants are present in fractions along the base-line and do not possess peroxidase activity.

Horseradish peroxidase was purified by the above procedure several times. In all cases high activity fractions were pooled and concentrated and generally referred to as isoenzyme C. The protein concentration of isoenzyme C was determined by the bicinchoninic acid assay. Concentrations obtained for various different batches of isoenzyme C were in the range of 20 - 60mg/l protein. Assays using various different concentrations of isoenzyme C read at various different intervals were performed in order to determine the optimum assay parameters for an isoenzyme standard assay. Fig. 3.3(a) and 3.3(b) show results of these assays. The standard conditions developed for the isoenzyme microassay were: isoenzyme concentration of 200 µg/l read at 620nm after 10 minutes using diluent and substrate conditions of HRP-TMB. The isoenzyme concentration used in this assay is higher than the enzyme concentration used in the TMB standard assay. This is because the
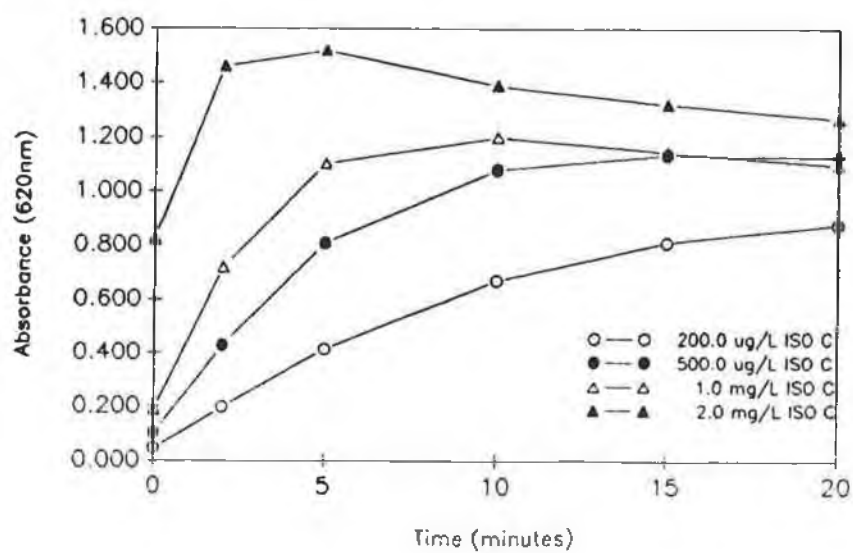
70

(a)
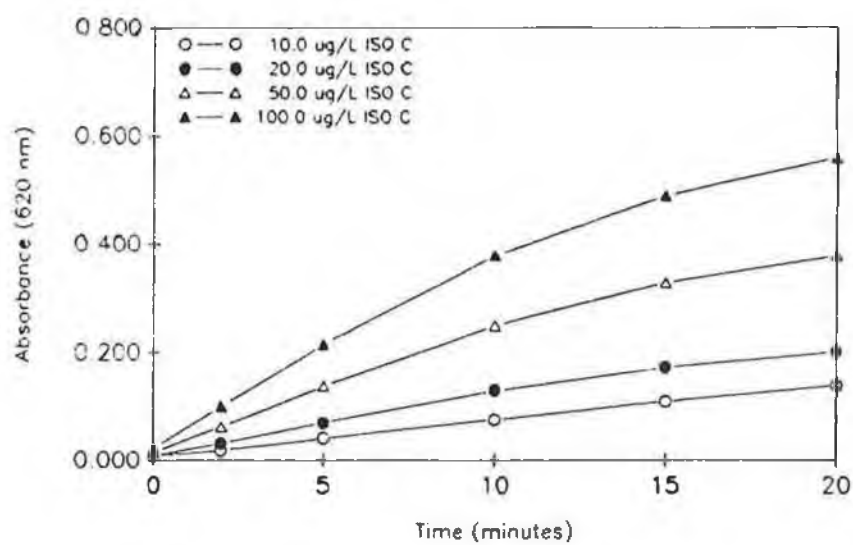
(b)

Fig 3.3 (a) and (b).   Absorbance vs. time for various isoenzyme C concentrations.

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isoenzyme shows less overall activity (after fraction were pooled and concentrated) than the native enzyme. After 10 minutes a desirable absorbance value was obtained and so the incubation time of 10 minutes was used in the isoenzyme standard assay.

The purification of isoenzyme C of horseradish peroxidase for use in preparation of enzyme - immunoglobulin conjugates is justified as the efficiency of enzyme conjugation to immunoglobulin will be increased and the immunoassay in which the conjugate is to be used will be more sensitive. HRP will have higher specific activity than commercial preparations and will be more homogenous after purification. Also, background staining will be lowered and use of antiserum will be more economical (Tijssen & Kurstak, 1984). However, for protein modification work (as will be seen in Chapter 4) the use of isoenzyme C was a disadvantage. No successful modification was seen with isoenzyme C probably due to the low protein content of the isoenzyme. The maximum yield of isoenzyme C after concentration from any batch was 8ml. This aliquot was quickly used up in any protein determination, assay standardization or chemical modification work. It was decided that HRP purification was too time-consuming relative to what it yielded. Therefore, chemical modification was mainly carried out directly on commercial Sigma type VI HRP. Some characterization of isoenzyme C was performed as described in Sections 3.2.2.2 and 3.3.

3.2.2.2.   SDS-Polyacrylamide Gel Electrophoresis:

SDS-polyacrylamide gel electrophoresis of native HRP and isoenzyme C was performed as described in Section 2.4. This was performed in order to establish the purity of commercial HRP and isoenzyme C. The protein stain used to identify bands on the gel was Coomassie brilliant blue (R-250 form). When staining for protein with Coomassie brilliant blue a protein concentration of at least 30µg/ml is required. The molecular weight of HRP is close to 44,000 (Welinder, 1979). Only one protein band corresponding to a molecular weight of approx. 44,000 - 48,000 was seen on gel after staining (not shown). This band was quite diffuse and

contained one large, discrete band and several smaller, more diffuse minor bands. These individual smaller bands within the large disperse band are probably the various isoenzymes of HRP present in commercial preparations (See Section 3.2.2.1). In the lanes where isoenzyme C was applied there is no banding seen. The concentration of applied isoenzyme C was estimated to be 60µg/ml so this was concentrated enough to be picked up by Coomassie protein stain. However, as no banding for isoenzyme was seen the most likely explanation is that the protein concentration was too low.

The large diffuse band obtained for the commercial preparation of horseradish peroxidase implies that the preparation applied was not very pure. This result is supported by the results from Section 3.2.2.1 where various peaks are seen on an elution profile of HRP. The disperse band, and the peaks of the elution profile as discussed in Section 3.2.2.1, are probably composed of the various isoenzymes of HRP (Shannon et al, 1966), some of which are highly active and others which are relative inactive. A non-denaturing gel, i.e., without the inclusion of sodium dodecyl sulphate, was also prepared at this stage. In a native gel the enzyme protein is not unfolded by a protein denaturant, neither are disulphide bonds broken by a reducing agent. Therefore the protein should remain active after the gel has been run. Substrates can be used to stain the gel for areas of activity. The procedure of Thomas et al (1976), which uses TMB to stain gels for peroxidase activity, was followed after Sigma HRP had been subjected to electrophoresis. However, no bands of activity could be distinguished.

3.2.3. Summary:

Commercial horseradish peroxidase was fractionated by ion exchange chromatography to yield the highly active, but rather low in protein, isoenzyme C. Other isoenzymes and various inactive proteins and contaminants were observed in the elution profile obtained for this procedure. SDS-polyacrylamide gel electrophoresis was performed to assess the purity of Sigma type VI HRP and of the

purified isoenzyme C. The commercial preparation contains several minor bands within one large disperse band. This does not point to a pure enzyme but to one that contains other components. In this case, as described in the literature, it is known that the components present are isoenzyme C and other isoenzymes.

The purification procedure was undertaken in an attempt to obtain a more active and homogeneous form of HRP for use in chemical modification experiments. However, the purification procedure was very time consuming relative to the yield in volume and protein content. Because of this the purification procedure was abandoned and isoenzyme C was used for only some chemical modification experiments. But the purification of an isoenzyme of HRP without the use of an ionic strength gradient may however be useful in other applications. Also, scale up of this procedure may be possible using an automated, reproducible, large scale protein purification system that would increase the yield of purified protein in volume and protein content.

3.3.   Thermal Stability of Horseradish Peroxidase:

3.3.1.   Introduction:

An assessment of the thermal stability of native horseradish peroxidase was undertaken prior to the commencement of any crosslinking and chemical modification experiments (These experiments will be the subject of Chapter 4). Chemical modification with crosslinking reagents can stabilize an enzyme against irreversible thermoinactivation. Reasons for this will be discussed in Chapter 4. The pattern of thermal denaturation and the inherent stability of the native enzyme must be established prior to any of these modification experiments. Once these properties of the native enzyme have been established, any changes in the thermal denaturation pattern of the native enzyme following modification can be monitored. Thermostability can be assessed by measuring residual activity of an enzyme that has been incubated at a particular temperature for a certain length of time (Ugarova et al, 1979, Torchilin et al, 1983, 1979 and 1978). Various other methods for

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measuring thermostability are available such as: measurement of melting temperature Tm, measurement of the protein free energy values, and estimation of the temperature of maximum stability. The retention of catalytic activity is an important stability index if modified HRP derivatives are to be used in biosensors or other applications. Measurement of residual activity at a particular temperature was chosen as it is easy to perform, is relatively quick and does not demand the use of expensive equipment. Various incubation temperatures were studied so that a temperature at which the enzyme inactivated at a conveniently measurable rate could be established. One particular temperature was chosen and the activity loss with time of HRP was studied to determine the reaction order of HRP activity loss. The kinetics of this thermal inactivation were calculated in order to determine what type of reaction order the thermal decay obeyed.

3.3.2. Results and Discussion:

3.3.2.1. Thermoinactivation of Horseradish Peroxidase:

Thermoinactivation of HRP at various temperatures was performed as described in Section 2.9. Thermoinactivation curves for native HRP samples incubated at 45°C to 75°C are shown in Fig.3.4. (a), (b) and (c). These curves are plotted as % relative catalytic activity vs. time. As can be seen from these decay curves, no thermal inactivation was seen at 45°C, i.e., no activity was lost over time. In fact an increase in catalytic activity is seen over the 100 minute incubation period. At 55°C an increase in catalytic activity is seen over time for approx. 60 minutes and then there is a fall in activity back to the original level at t = 0 (See Fig.3.4(a)). For samples incubated at 60°C, 65°C and 70°C there is an increase in catalytic activity seen for 35, 25 and 25 minutes respectively (See Fig 3.4 (b)). Then, enzyme activity levels begin to decrease. In the case of samples incubated at 60°C activity only falls to 50% of starting activity after 100 minutes. For samples incubated at 65°C and 70°C, 10% and 0% activity remain, respectively, after 100 minutes. It is unclear why there is an increase in catalytic activity for a certain period at the temperatures mentioned above. Apparent thermal activation, as opposed to the thermal inactivation that was
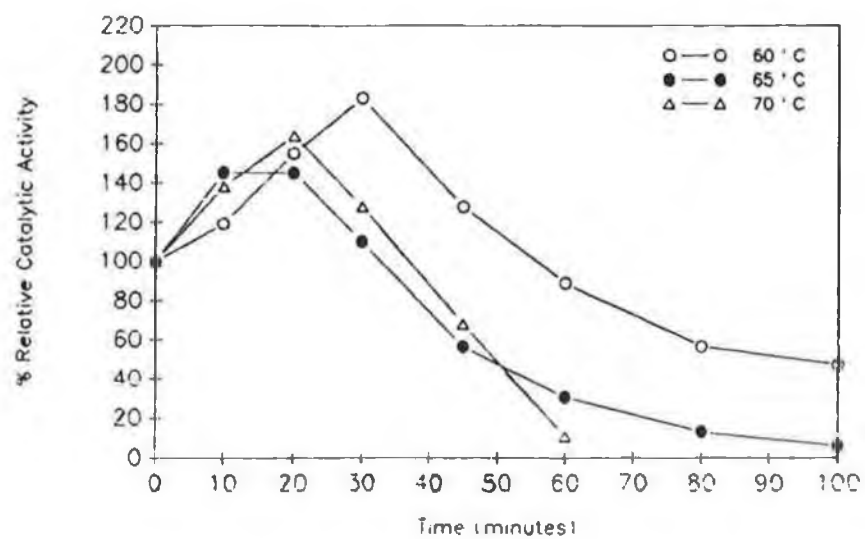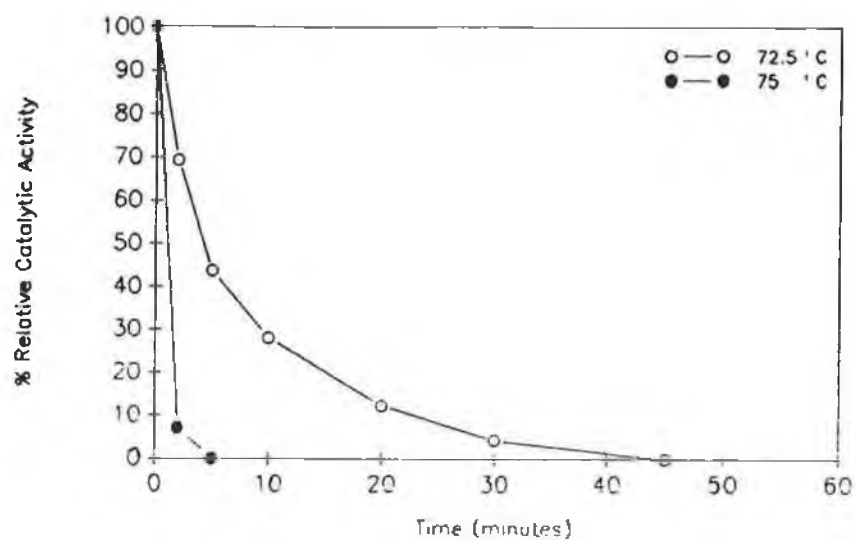
(a)
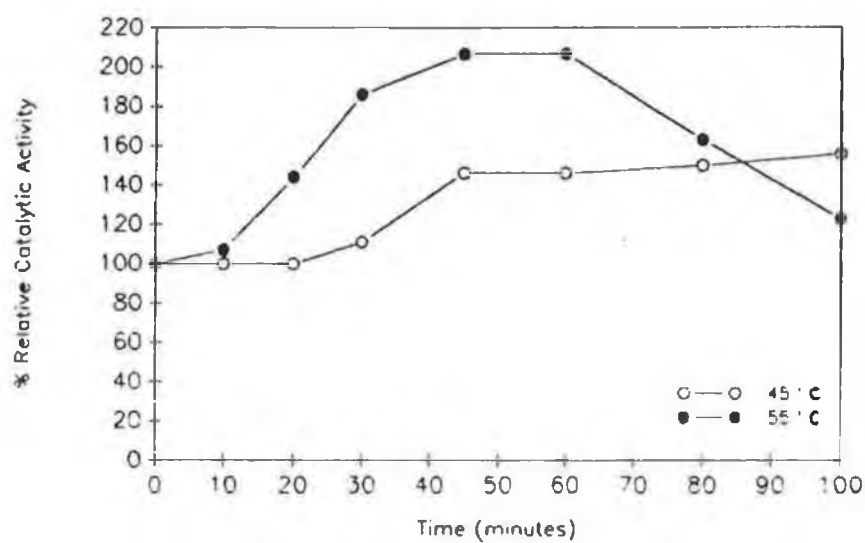
(b)

Fig. 3.4 (a) and (b).   Thermoinactivation curves for native HRP incubated at various temperatures.
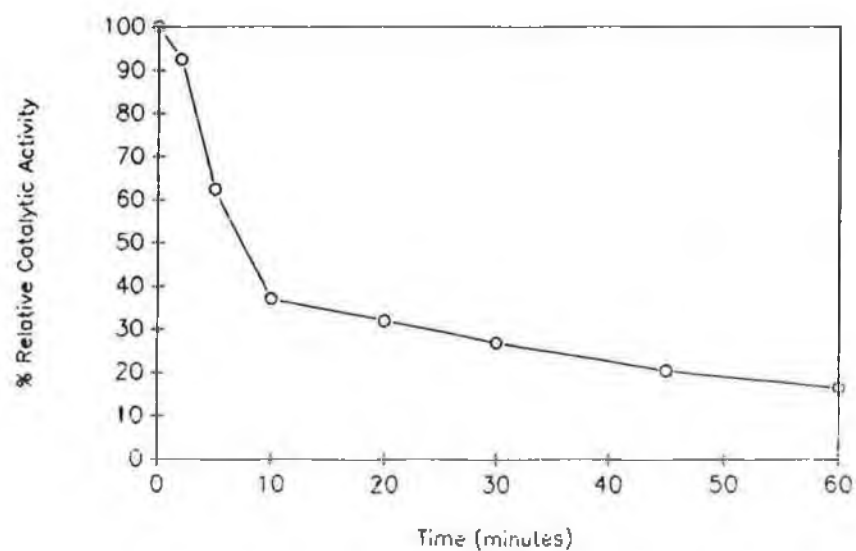
76

(c)

(d)

Fig. 3.4 (c) and (d). Thermoinactivation curves for native HRP incubated at various temperatures (c) and for isoenzyme C incubated at 72.5°C (d).

expected, is occurring. As the temperature increases the extent of this thermal activation decreases. This phenomenon has been reported for some immobilized and chemically modified enzymes (Henley & Sadana, 1985).

An enzyme denatures at an elevated temperature because unfolding of the protein backbone occurs. At high temperatures, non-covalent interactions, that usually maintain the native enzyme structure at moderate temperatures, are lost. Non-native non-covalent bonds are acquired by the enzyme at high temperatures. These irregular noncovalent interactions are unstable and remain during the heating process (Mozhaev & Martinek, 1982). Activity is lost if the heating is prolonged as unfolding disrupts the active, or functional, site to an extent that is irreversible. Irreversible enzyme thermoinactivation is the process that is responsible for the gradual loss of enzyme activity with time at an elevated temperature. It is possible that at elevated temperatures the peroxidase molecule has partially unfolded to yield a structural form that has enhanced activity. Weng et al (1991) studied the thermal stability of HRP and put forward two theories to explain the biphasic behaviour seen. The first is the two fraction theory originally described by Chang et al (1988). Chang et al reported a reaction order of 1.5 for peroxidase that they attributed to the presence of isoenzymes of differing thermal stabilities. The existence of heat-labile and heat-stable fractions explains biphasic behaviour in this theory. The second theory involves the formation of an intermediate during the heating process, that is partially denatured. This intermediate has a higher activity than the native enzyme. The second theory of Weng et al explains the activation seen for HRP in this case. Ugarova et al (1979) also studied the thermoinactivation kinetics of horseradish peroxidase during the course of modification experiments. A biphasic or non-first order reaction rate observed by them may be due to heterogeneity of preparations studied, especially modified preparations. The rate at 60° C observed by Ugarova et al appeared first order initially, and then assumed a non-first order rate. The second order reaction rate seen above for HRP may be due to impure preparations, even the relatively pure isoenzyme C, being studied. Loss of activity could take place via two distinct molecular processes, rather than by one, thereby giving the second order rate.

As can be seen in Fig. 3.4 (c), at 72.5°C an even decay rate is observed for incubated enzyme samples over time. At this temperature there is no apparent activation and no sharp decrease in activity seen with time. 75°C was the highest temperature at which enzyme inactivation was carried out. As can be seen from the thermal decay curve in Fig.3.4 (c), all of the enzyme activity is lost after 5 minutes at this temperature. A temperature between 72.5°C and 75°C was chosen as the temperature at which to study enzyme activity loss with time. The enzyme inactivates at a conveniently measurable rate in this range and so it is ideal for studying thermal inactivation. This is the temperature at which the thermal stability of modified derivatives was studied (See Chapter 4.0). As can be seen from Fig 3.4 (a), (b) and (c), the rate of loss of % relative catalytic activity over time increases as the temperature increases. The apparent thermal activation effect is not evident at the higher temperatures.

Isoenzyme C was incubated at 72.5°C and thermoinactivation was observed as described in Section 2.9. The concentration of isoenzyme C incubated was 60 mg/l. The thermal decay curve for isoenzyme C is shown in Fig 3.4 (d). The decay appears uniform over time as is the case for native HRP above. No incubations at other temperatures were performed as 72.5°C is a suitable temperature at which to observe thermoinactivation of isoenzyme C.

3.3.2.2. Kinetics of Thermoinactivation of Horseradish Peroxidase:

The kinetics of thermoinactivation of horseradish peroxidase and isoenzyme C were studied at 72.5°C as described in Section 2.10. The results of % relative catalytic activity vs. time were analyzed using the computer programme, Enzfitter. Data was fitted to first order and second order exponential equations to determine if decay is first or second order, i.e., if activity loss is consistent with unimolecular or with a more complex mechanism (See Fig 3.5 (a) and (b) and 3.6 (a) and (b)). Visual observation of the graphic fit shows that both HRP and isoenzyme C thermal inactivation obey second order rate equations.
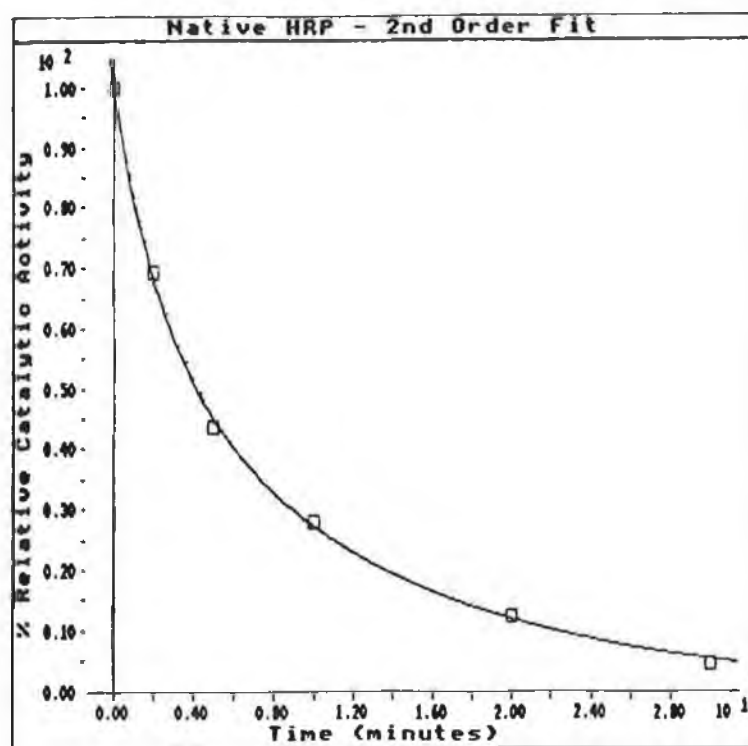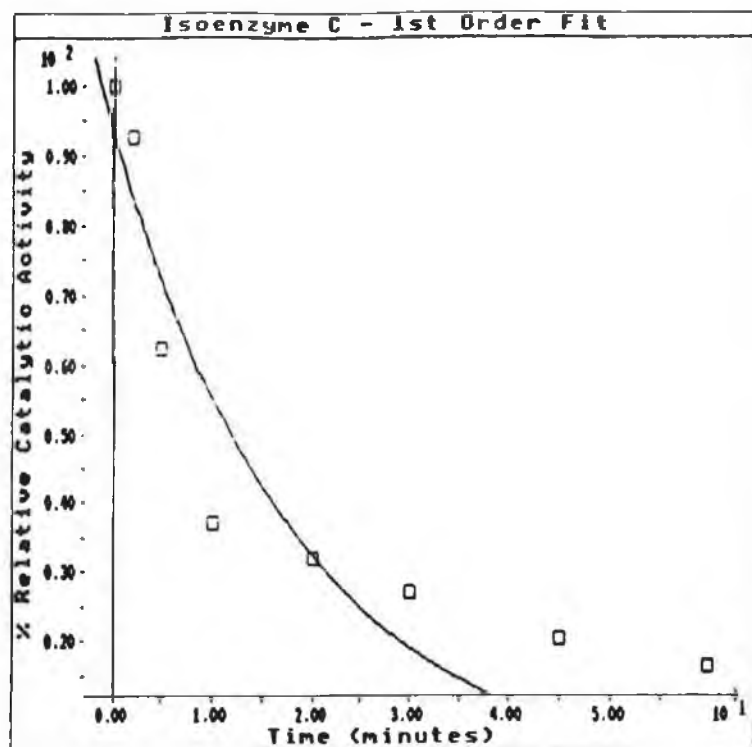
(a)
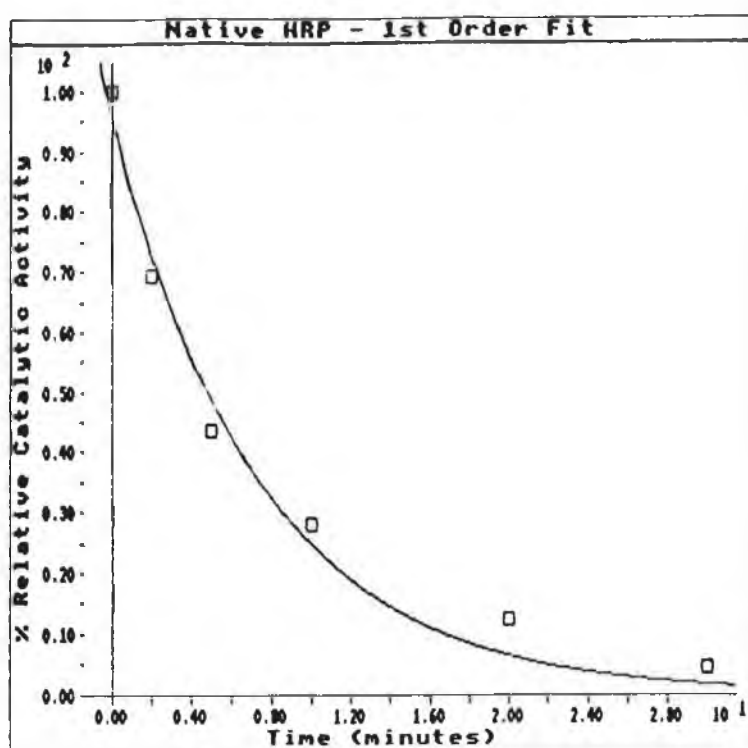
(b)

Fig. 3.5 (a) and (b). Native HRP fitted to first- and second-order exponential equations
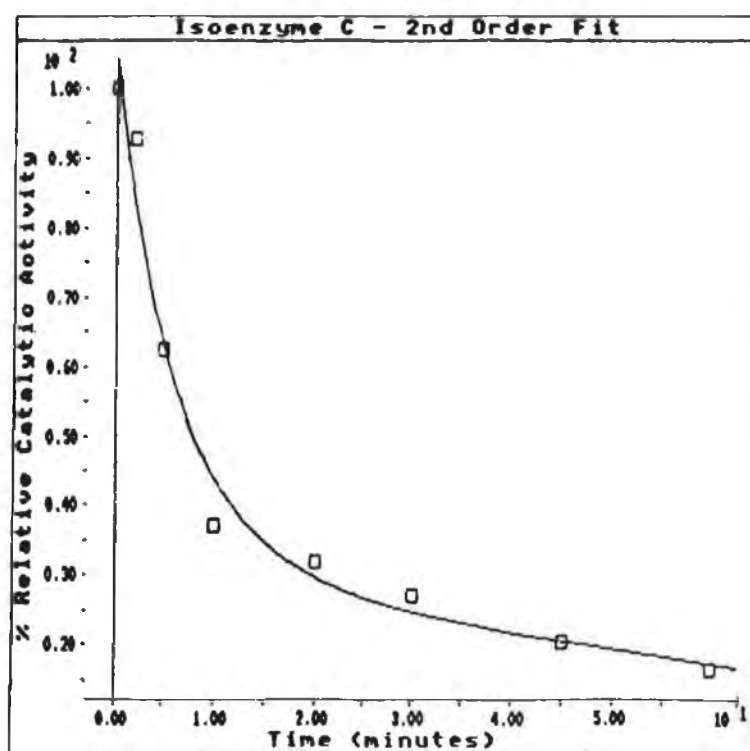
80

(a)

(b)

Fig. 3.6 (a) and (b). Isoenzyme fitted to first- and second-order exponential equations.

The kinetics of enzyme thermoinactivation obey either zero, first, second or pseudo-first order kinetic laws but often deviate from first order (Melik-Nubarov et al, 1987, O'Fagain et al, 1991a). It has been found that HRP follows a second order rate equation. The Arrhenius equation can be used to determine activation energies from first-order kinetic data. From derived rate constants, the useful shelf-life of an, e.g., modified enzyme, can be determined at a temperature of interest. Predictions of this sort are referred to as accelerated storage studies. As will be discussed in Chapter 4.0, the aim of chemical modification experiments is to stabilize the enzyme concerned. Enhanced stability will be seen in increased stability of the modified protein towards heat denaturation and also in prolonged shelf-life, relative to the unmodified form. Accelerated storage studies can be used in chemical modification or protein engineering studies to assess the shelf-life of the modified derivative. However, because HRP and isoenzyme C obey second order kinetics, extrapolations of data by the Arrhenius equation are invalid.

3.3.3. Summary:

The thermal stability of native horseradish peroxidase was studied. The stability of the native enzyme towards heat denaturation was studied by observing thermoinactivation at various different temperatures. At lower temperatures an apparent thermal activation resulted. At higher temperatures, thermal inactivation was seen. 72.5°C was the temperature decided upon at which to carry out thermoinactivation studies of modified derivatives in future modification experiments. At this temperature, there is an even loss of enzyme activity over time. This decay obeys a second order reaction rate, as revealed by fitting of the data using Enzfitter. The fact that the decay of HRP at 72.5°C is second order means that accelerated storage studies using the Arrhenius equation to predict shelf-life cannot be undertaken.

4. CHEMICAL MODIFICATION STUDIES

4.1. Crosslinking with Bis-imidates:

4.1.1. Introduction:

Bis-imidates are homobifunctional crosslinking reagents with an imidoester as the reactive functional group, one at either end of the molecule. These diimidoesters are amino specific and can be used to crosslink proteins. The three bis-imidates used in this Section are: dimethyl suberimidate, dimethyl pimelimidate and dimethyl adipimidate. The dihydrochloride, water soluble form of these reagents was used. Bis-imidates react with α- and ε-amino groups in proteins (Hunter & Ludwig, 1972). By appropriate choice of pH, either α- or ε-amino groups can be selected for. The general structure of a diimidoester is shown in Fig.4.1.

$$Cl^{-}\ H_2\overset{+}{N}\!\!=\!\!\underset{H_3CO}{\overset{}{C}}\!-\!(CH_2)_n\!-\!\underset{OCH_3}{\overset{}{C}}\!=\!\overset{+}{N}\ H_2\ Cl^{-}$$
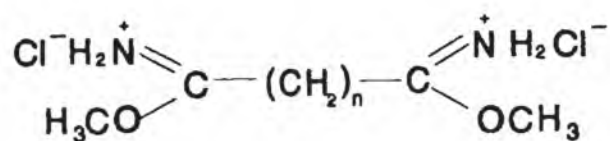
Fig.4.1.   Chemical formula of a diimidoester.

The bis-imidates vary mainly in the length of the spacer alkyl chain which separates the imidoester functions. The "n" value designates the number of $-CH_2-$ groups and varies for each bis-imidate. For dimethyl suberimidate $n = 6$, for dimethyl pimelimidate $n = 5$ and for dimethyl adipimidate $n = 4$ (Han et al, 1984). The higher the n number, the greater the distance the crosslinker can span. The crosslinking distance of a bis-imidate will have an effect on the success of crosslink formation. The length of the crosslinks or molecular bridge that are formed by dimethyl suberimidate, dimethyl pimelimidate and dimethyl adipimidate are 11Å, 10Å and 9Å respectively (Ji, 1983). A bis-imidate shorter than 5Å usually yields few or no crosslinks, whereas extensive crosslinking can frequently result when the length of the crosslinker is 11Å or higher. However, a greater increase in crosslinker

length may not be advantageous (Ji, 1983). It is hoped that intramolecular crosslinks will be formed that will stabilize the tertiary structure of HRP. Oligomers formed due to intermolecular crosslinks may also lead to increased stability of the protein. A modified enzyme may be more resistant to heat denaturation than the native enzyme. Crosslinking prevents unfolding of the protein backbone. Loss of the functional three dimensional structure of an enzyme usually occurs upon heating. Because a modified enzyme has a rigidified structure, the active conformation should not be lost when a modified enzyme is heated. Increased thermostability is taken as an indication that crosslinking has occurred. Monofunctional reagents, i.e., with one reactive end, may be used to distinguish between true crosslinking and one-point modification.

Bis-imidates are readily soluble in aqueous buffers. They react under mild conditions, i.e., pH 7.0 - 10.0 at room temperature and have a high degree of specificity for primary amino groups (Ji, 1983). The 6 lysine groups per HRP molecule (Welinder, 1979) are the targets for the bis-imidates used in this Section. The side reaction of bis-imidates with thiol, phenolic, carboxyl, imidazolyl and guanidyl groups are negligible. Lysine reacts with a bis-imidate at high pH values. The result of the reaction of a primary amine with an imidoester function is an amidine linkage, which is quite stable. By formation of this crosslink, two amino groups are joined via an imidate molecule. The positive charge on the original amino group is retained. The fact that the amidine group formed has similar charge characteristics to the original amine probably explains the fact that amidination leads to only minimal effects on protein structure (Inman et al, 1983) and on catalytic activity of the targeted enzyme.

The bis-imidate modification reaction, an amidination reaction, is depicted in Fig.4.2 (O'Fagain et al, 1991a). In this Section various reaction conditions were tested in order to obtain optimum crosslinking conditions for the bis-imidates. When stabilization was observed, the effect was studied over a number of days. In the cases where stabilization persisted at a high level over a number of days, changes in other properties of the modified enzyme were investigated.
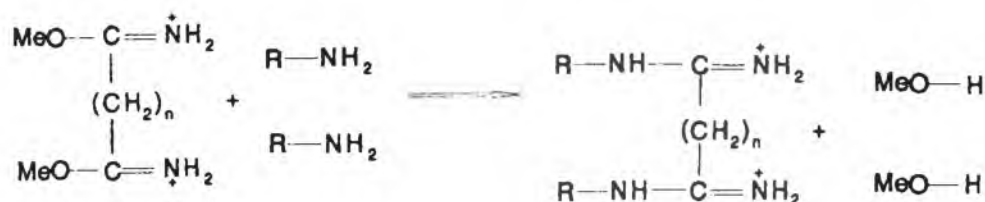
84

MeO—C=ṄH₂
|
(CH₂)ₙ   +   R—NH₂
|
MeO—C=ṄH₂   R—NH₂

⟶

R—NH—C=ṄH₂   MeO—H
|
(CH₂)ₙ   +
|
R—NH—C=ṄH₂   MeO—H

Fig. 4.2.   Bisimidate modification reaction.

4.1.2.   Results and Discussion.

4.1.2.1.   Crosslinking with Dimethyl Suberimidate:

Crosslinking of horseradish peroxidase with dimethyl suberimidate was performed as described in Section 2.5.1. The structure for dimethyl suberimidate dihydrochloride is shown in Fig. 4.3. Dimethyl suberimidate has been used extensively in enzyme modification because of its ability to span a long distance.

$$Cl^-H_2\overset{+}{N} \!\!\diagdown_{C}\!\!\diagup^{H_3CO}\!\!-(CH_2)_6-C\diagup^{\overset{+}{N}H_2Cl^-}_{OCH_3}$$
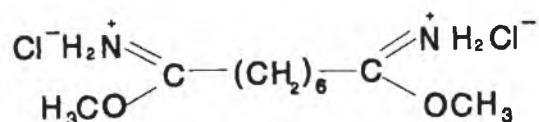
Fig. 4.3.   Structure of dimethyl suberimidate.

A wide range of dimethyl suberimidate (DMS) concentrations were used at two different pH values in order to optimize the procedure for crosslinking horseradish peroxidase with DMS. It was discovered that the ratio of protein to DMS was important, not only in concentration terms, but also in volume : volume ratio. Minotani et al, (1979) performed an imidate crosslinking reaction for 1 hour at room

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temperature, so these conditions were adopted here. Dimethyl suberimidate is unstable in solution (De Renobales & Welch, 1980) and is often used as a dry powder for effective crosslinking. However, if DMS (and any other imidate) is prepared in solution and used immediately there should be no reagent instability problems and also saturation effects are less likely (Hunter & Ludwig, 1972). For all crosslinking reactions DMS was prepared in 0.1M phosphate buffer pH 8.0. Horseradish peroxidase was prepared at a concentration of 1 mg/ml in either 0.1M phosphate buffer, pH 7.0 or pH 8.0. The pH range for the imidate reaction is pH 7.0 - 10.0. At a high pH, amidination is favoured over reagent hydrolysis (Hunter & Ludwig, 1972). A high degree of crosslinking was seen when pH 7.0 was used.

The crosslinking reaction was allowed to proceed for 1 hour at room temperature. De Renobales & Welch (1980) and Sheehan et al (1990) terminated the bis-imidate reaction by gel filtration of the reaction mixture through Sephadex G-25. Minotani et al (1979) terminated the crosslinking reaction by dialysis and O'Fagain et al (1991a) used Tris-HCl as a stopping reagent. The crosslinking reaction as described in Section 2.5.1 was considered complete after 1 hour. Therefore, it was not thought necessary to terminate the reaction as any unreacted dimethyl suberimidate had probably hydrolysed after 1 hour.

Modified samples were assayed after the crosslinking reaction for initial recoveries as described in Section 2.5.1. For all samples it was found that no loss of the original activity had occurred. For all concentrations of DMS used there was 100% recovery of activity, relative to native unmodified samples. After assaying for initial activities, the thermoinactivation of native and modified HRP was studied as described in Section 2.5.1. At lower DMS concentrations, i.e. 250 µg/ml and lower, no enhanced thermostability is seen relative to native HRP. Thermoinactivation studies reveal that HRP modified with DMS at concentrations from 0.1 mg/ml to 5.0 mg/ml are more stable towards heat denaturation than the native enzyme. Therefore, it is taken that crosslinking has occurred at these concentrations. Fig. 4.4 shows the thermoinactivation at 72.5°C of HRP crosslinked with DMS. As can be seen, native HRP decays at a rate similar to that seen in Section 3.3.2.1.
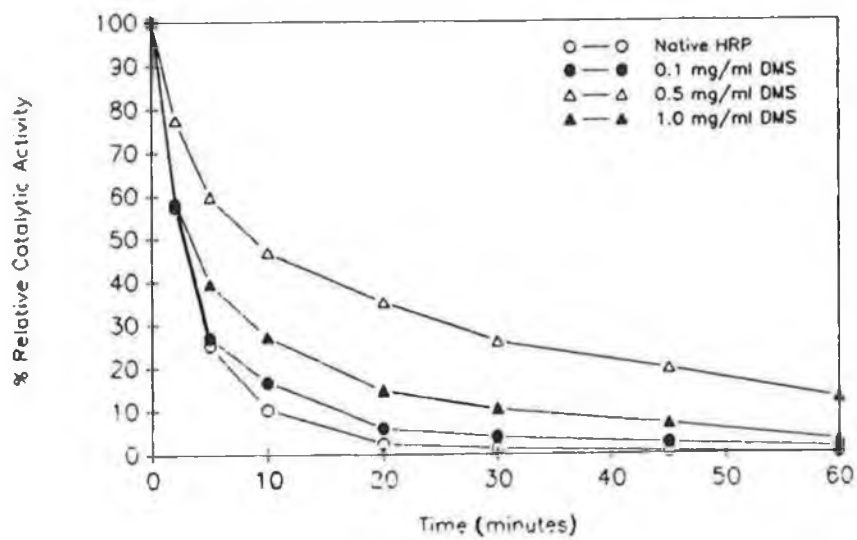
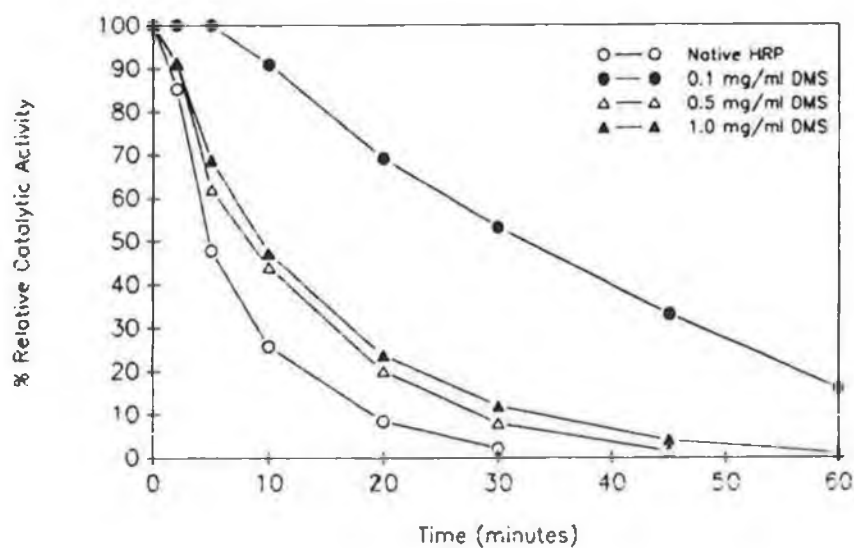Fig. 4.4.   Thermoinactivation at 72.5°C of HRP treated with DMS.

Fig. 4.5.   Thermoinactivation at 72.5°C of 1 day old DMS modified samples.

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Over the 60 minute incubation period, the 0.5 mg/ml DMS sample appears to be the most stable towards heat denaturation, relative to native HRP. The 0.5 mg/ml DMS sample retains 12 % of its activity after 60 minutes whereas the native enzyme has lost all its activity. The 1.0 mg/ml DMS sample, and to a lesser extent the 0.1 mg/ml DMS sample, show some enhanced thermal stability initially but these samples retain only 3% and 1% respectively of original enzyme activity after 60 minutes. Crosslinking at these DMS concentrations can only be achieved if a critical set of conditions is followed. If HRP in 0.1M phosphate buffer pH 8.0 is used in modification reaction then no crosslinking occurs. Also, the concentration of HRP is critical, i.e. concentrations outside the range used above do not yield any samples with enhanced thermostability. The volume of protein : crosslinker is also very important. A 50 : 1 ratio, i.e. 250µl HRP to which is added 5µl DMS solution, must be used if any crosslinking is to occur. This volume ratio ensures that protein is in excess.

An apparent increase in the stability towards heat denaturation of the modified samples is seen over a 1 day period. Fig. 4.5 shows the thermoinactivation at 72.5°C over 60 minutes of 1 day old DMS modified sample. Overall the 0.1 mg/ml DMS sample shows the greatest degree of thermostability. The effect seen for this sample is more pronounced than it was on the day of modification, i.e., 1 day previously. The 0.5 mg/ml DMS sample seems to have lost some of its thermal stability whereas the thermostability of the 1.0 mg/ml DMS sample remains the same. Table 4.1 (p. 91) compares the apparent half-lives ($t_{\frac{1}{2}}$) for the samples shown in Fig. 4.4 and Fig. 4.5, i.e., the time required for the activity to fall from 100% to 50%. These are apparent and not true half-lives since HRP activity loss is not first-order. It is not clear why some of the samples are more thermostable 1 day after crosslinking was performed. This effect could be due to the phenomenon of " selfstabilization " (Torchilin et al, 1979). Selfstabilization of the enzyme occurs as the enzyme "selects" the most stable crosslink, typically from a range of crosslinkers of different length. In this case of dimethyl suberimidate acting on HRP, crosslinks formed initially may reverse and reform in a different, more stabilizing position on the HRP molecule.

The increase in thermal stability for HRP crosslinked with DMS from those levels seen on day of modification to levels seen 1 day afterwards, is not maintained after 4 days. In fact, there is no enhanced thermostability evident for any of the samples (See Fig. 4.6). DMS treated samples were more stable than native HRP on the day of the modification reaction and 1 day afterwards. Native HRP is the most thermostable sample seen 4 days after modification was carried out. The decay seen for native HRP is the characteristic pattern seen in Section 3.3.2.1. Any crosslinks formed by DMS, that were evident due to increased thermostability of modified samples, seen in Fig. 4.4 and Fig. 4.5, are reversible and short-lived. An attempt was made to render the DMS crosslinks formed during modification reaction more permanent and irreversible. Sodium borohydride treatment of modified samples was carried out as described in Section 2.7.1. Tijssen (1985) uses sodium borohydride to reduce unstable amino-carbonyl bonds that are formed when a peroxidase is conjugated to an antibody. The use of sodium borohydride stabilizes the enzyme - antibody conjugates so that these conjugates may be used in immunoassays and other applications. This strategy was applied to DMS crosslinked HRP, which also contains amino-carbonyl bonds. Fig. 4.7 depicts the thermal decay at approx. 72.5°C of native and DMS modified samples that were treated with borohydride on the day of modification. These samples were prepared 4 days before performance of this thermostability assay. Table 4.2 (p. 91) compares the half-lives of modified and borohydride-modified HRP. Looking at Table 4.2 and comparing Fig. 4.7 to Fig. 4.6 it is evident that the use of borohydride has fixed the crosslinks in position and has prevented them from reversing.

Isoenzyme C, purified in Section 2.3, was also modified with dimethyl suberimidate in an attempt to crosslink the isoenzyme. DMS concentrations used ranged from 0.1 mg/ml to 5.0 mg/ml. The protein concentration of isoenzyme C in the reaction mixture was 20 - 60 mg/l and its pH 7.0. A protein : crosslinker ratio of 50 : 1 and also 1 : 1 were used. No enhanced thermostability was seen for any isoenzyme samples that had been treated with DMS. In other words, no crosslinking of isoenzyme C occurred at any of the concentrations or ratios used. Horseradish peroxidase was purified to yield isoenzyme C in Section 2.3 in order to obtain a
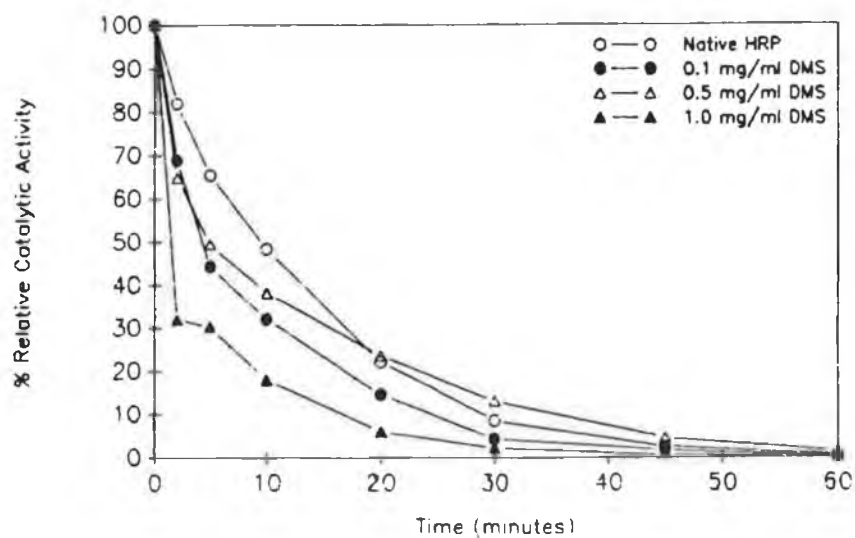
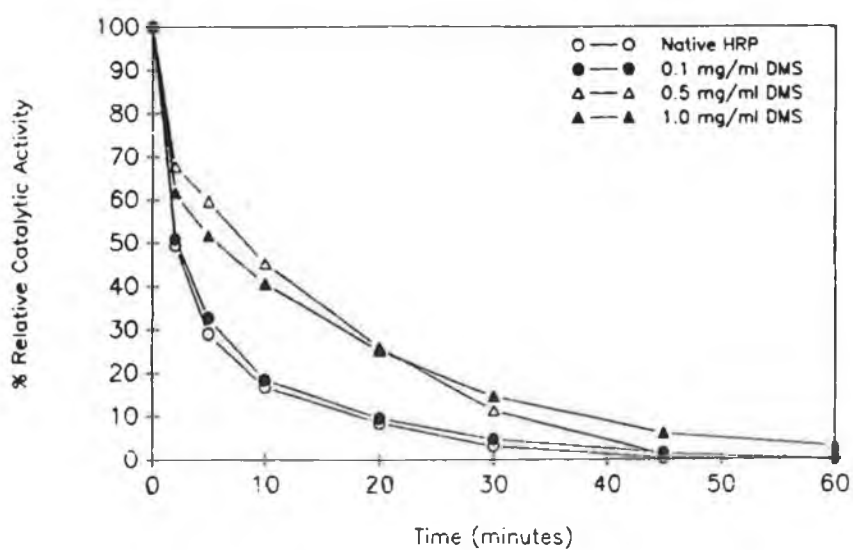Fig. 4.6.   Thermoinactivation at 72.5°C of four day old DMS treated samples.

Fig. 4.7.   Thermoinactivation at 72.5°C of 4 day old borohydride treated DMS samples.

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Table 4.1.   Table of apparent half-lives of HRP samples from Figs. 4.4 and 4.5.

"DAY 0" (FIG. 4.4.)	$t_{1/2}$ (min)	"DAY ONE" (FIG. 4.5.)	$t_{1/2}$ (min)
Native HRP	3	Native HRP	6
0.1 mg/ml DMS	3	0.1 mg/ml DMS	33
0.5 mg/ml DMS	9	0.5 mg/ml DMS	9
1.0 mg/ml DMS	4	1.0 mg/ml DMS	10

Table 4.2.   Table of apparent half-lives of HRP samples from Figs. 4.6 and 4.7.

"DAY 4" (FIG. 4.6.)	$t_{1/2}$ (min)	"DAY 4 REDUCED" (FIG. 4.7.)	$t_{1/2}$ (min)
Native HRP	10	Native HRP	3
0.1 mg/ml DMS	4	0.1 mg/ml DMS	3
0.5 mg/ml DMS	5	0.5 mg/ml DMS	9
1.0 mg/ml DMS	2	1.0 mg/ml DMS	8

Table 4.3.   Table of concentration of amino group of centrifuged DMS stocks.

SAMPLE[*]	O.D. $_{420}$	Conc. (mM)
Native HRP	0.079	0.245
0.5 mg/ml DMS	0.058	0.184
1.0 mg/ml DMS	0.011	0.044

[*] Results for 0.1 mg/ml DMS sample not available.

highly pure and active, uncontaminated form of HRP that could successfully be crosslinked. However, no crosslinking of isoenzyme C occurred, probably because the protein content of the isoenzyme was too low. Higher molar ratios than were used here would need to be used to crosslink isoenzyme C with DMS. Because successful results were obtained using Sigma type VI HRP, crosslinking of isoenzyme C was not pursued further.

Thermal stability is not the only characteristic of an enzyme that may be altered upon modification. In recent years, quantitative data on the extent of modification has become more attainable due to the advancement of techniques such as radiolabeleling of crosslinking reagents, ion-exchange and gel exclusion chromatography and the use of automated amino acid analyzers (Means & Feeney, 1990). All these techniques allow monitoring of the effects of modification on relatively small amounts of protein. However, many of these techniques are time consuming and involve the use of expensive equipment and trained personnel. A simple and quick method by which a modified protein, that has been treated with an amino specific modifier, can be characterized is by the trinitrobenzenesulphonate (TNBS) assay for amino group determination (Hunter & Ludwig, 1972). This titration of amino groups using TNBS is based on the method of Fields (1971). Modified samples were reacted with TNBS as described in Section 2.6. The number of free amino groups remaining after amidination can be measured using this assay. A DMS crosslinked enzyme should have less amino groups present than an unmodified enzyme. Several attempts to titrate the number of amino groups on DMS-HRP yielded results in which the modified samples had higher levels of amino groups than native HRP. It would appear from these results that unbound/unreacted DMS is giving rise to the high results seen for modified stocks. In an attempt to remove any unbound DMS or interfering substances, modified stocks were centrifuged according to Section 2.8 and centrifuged samples were then assayed for amino group content. Modified, centrifuged stocks showed lower values for amino group content than native sample (See Table 4.3). So, it would appear that unbound DMS and any other interfering substances had been removed. Although the values are quite low overall for amino groups, there seems to be some correlation between

modified stocks and amount of amino groups. In theory, there should be a direct correlation between crosslinker concentration and value for amino groups, i.e. the more pronounced the crosslinking effect seen in terms of thermostability, the lower the value for amino groups should be. However, the results obtained here are not conclusive.

The enhanced thermostability that is seen in DMS treated enzyme (and in other amidinated proteins) has been attributed to two causes by Minotani et al (1979). The reasons are the covalent bridge formation between pairs of lysine residues and the shift in pK value of the lysine residues to higher values after acetamidination. The most likely reason for enhanced thermostability is probably due to the prevention of unfolding of the crosslinked protein backbone (Ugarova et al, 1979).

4.1.2.2.   Crosslinking with Dimethyl Pimelimidate:

Crosslinking of horseradish peroxidase with dimethyl pimelimidate (DMP) was performed as described in Section 2.5.2. The structure of dimethyl pimelimidate is shown in Fig. 4.8.
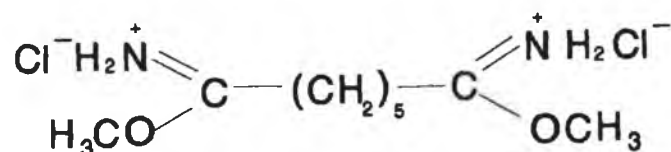
Fig. 4.8.   Structure of dimethyl pimelimidate.

Various concentrations, buffers, reaction pH and protein : modifier ratios were tested to determine suitable crosslinking parameters. Following on from dimethyl suberimidate crosslinking experiments, a narrower range of DMP concentrations was used. The range used was 0.1 to 10.0 mg/ml DMP. HRP concentration was 1 mg/ml in phosphate buffer pH 7.0 or 8.0. Details of reaction optima and conditions cited for DMS also apply to DMP. HRP was stabilized towards heat denaturation

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when the following conditions were used: 1 mg/ml DMP in phosphate buffer pH 8.0, 1.0 mg/ml HRP in phosphate buffer pH 7.0, a protein to modifier molar ratio of 50 : 1 (See Fig. 4.9). The optimum reaction pH for DMP cited in the literature is pH 8.0 (Ji, 1983). It was found in this case that if HRP pH 7.0 was used, in conjunction with DMP pH 8.0, then stabilized derivatives were obtained.

However, the increased thermostability seen in Fig. 4.9 is evident only for approx. 20 minutes. It is possible that the crosslinks formed are able to withstand heating for only 20 minutes, after which time they break. An increase in DMP concentration did not lead to an increase in the thermostability of HRP. Although stabilization was seen at higher concentrations, specifically at 5.0 mg/ml DMP, the effect was more pronounced for 1.0 mg/ml DMP. It appears that there is a critical DMP concentration for crosslinking, i.e. there is only one concentration at which effective crosslinking will occur. Sheehan et al (1990) found that there is an optimum concentration in DMS crosslinking. This also appears to be the case in DMP crosslinking. The crosslinking of a protein molecule is a very specific procedure and only one set of assay conditions and a very narrow range of crosslinking lengths will bring about successful modification. Other experiments involving isoenzyme C and other assay conditions and buffers, such as triethanolamine-HCl, pH 8.0 (as recommended by Hunter & Ludwig, 1972) yielded no samples that showed enhanced thermostability. As was the case with DMS, no adverse effect on HRP activity was seen following reaction with DMP. Overall, DMP modification was not as successful as DMS modification.

For DMP samples assayed 1 day after modification experiment was performed, greater stability was seen towards heat denaturation than was evident on day of modification experiment. Despite literature reports of a rapid reaction rate, it is possible that the reaction had not gone to completion in one hour. The effect of selfstabilization, as discussed in Section 4.1.2.1, is probably responsible for this. The 1 mg/ml DMP sample, which was the most thermostable on day of reaction, still shows enhanced thermostability but the 0.1 mg/ml DMP sample has far greater thermostability. On the day of modification the 0.1 mg/ml DMP sample did not
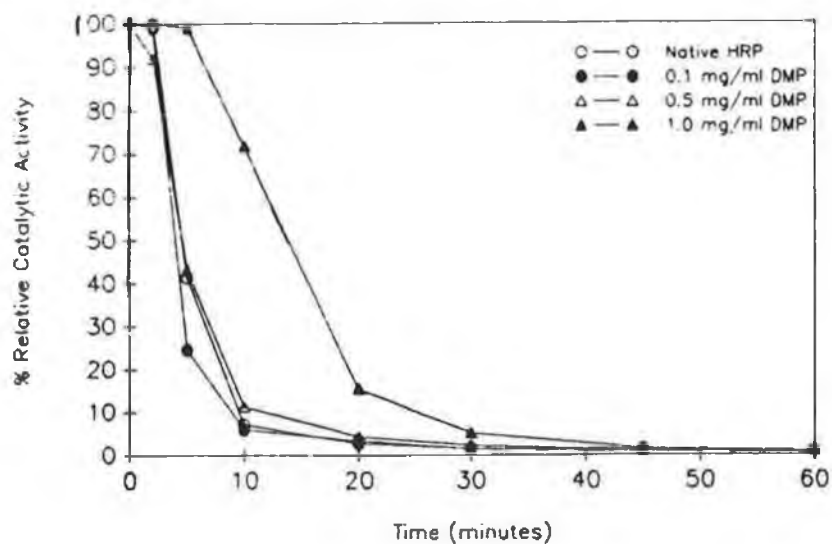
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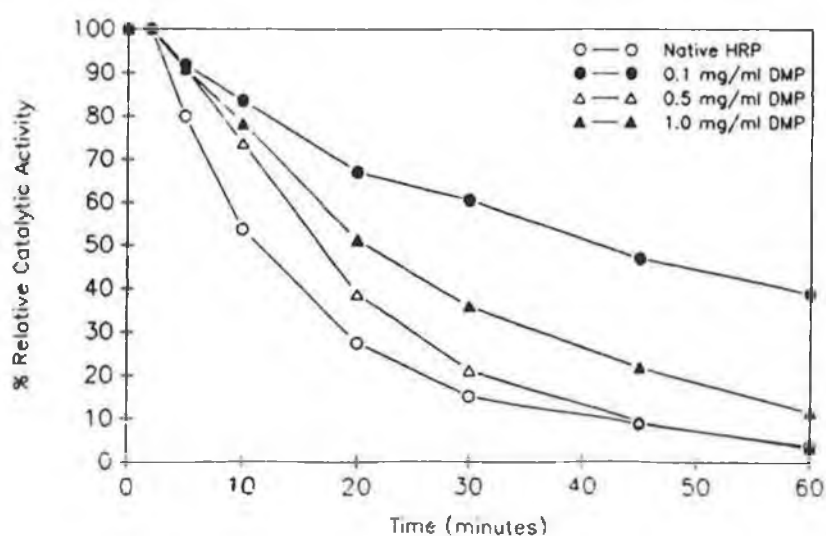Fig. 4.9.   Thermoinactivation at 72.5°C of DMP treated HRP samples.

Fig. 4.10.   Thermoinactivation at 72.5°C of one day old DMP samples.

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show any enhanced thermostability. One day later the 0.1 mg/ml DMP sample retains 38% of the original activity after 60 minutes incubation at approx. 72.5°C. (See Fig. 4.10) and shows enhanced thermostability throughout the incubation period. Borohydride treatment of DMP-HRP, in an attempt to reduce the crosslinks present in Fig.4.10, was performed as described in Section 2.7.2. The procedure used was adapted from one used by Blanco & Guisan (1989), in which borohydride was used to reduce Schiff bases formed when trypsin was covalently attached to agarose gels. Unstable Schiff bases result at points of crosslinking and the use of borohydride helps stabilize the crosslinks and prevents them from reversing. Fig. 4.11 shows the thermal decay of DMP samples that were modified 3 days previously. All pronounced thermostable effects, that were present in Fig. 4.10 have disappeared. From this it is presumed that all crosslinks have reversed. Although the 0.1 mg/ml DMP sample shows slightly more resistance to heat denaturation than the native enzyme, this is negligible when compared with the enhanced thermostability seen in Fig. 4.10. The thermoinactivation of borohydride treated DMP stocks, that were treated with borohydride two days previously, is depicted in Fig. 4.12. The thermal decay pattern is similar to that of untreated DMP stocks shown in Fig 4.11. It appears that borohydride has had no effect, either good or bad, on DMP samples. Table 4.4 gives a comparison of the apparent half-lives for all DMP stocks.

4.1.2.3.   Crosslinking with Dimethyl Adipimidate:

Crosslinking of horseradish peroxidase with dimethyl adipimidate (DMA) was performed as described in Section 2.5.3. The structure of DMA is shown in Fig. 4.13.
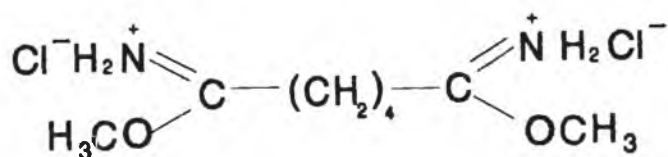

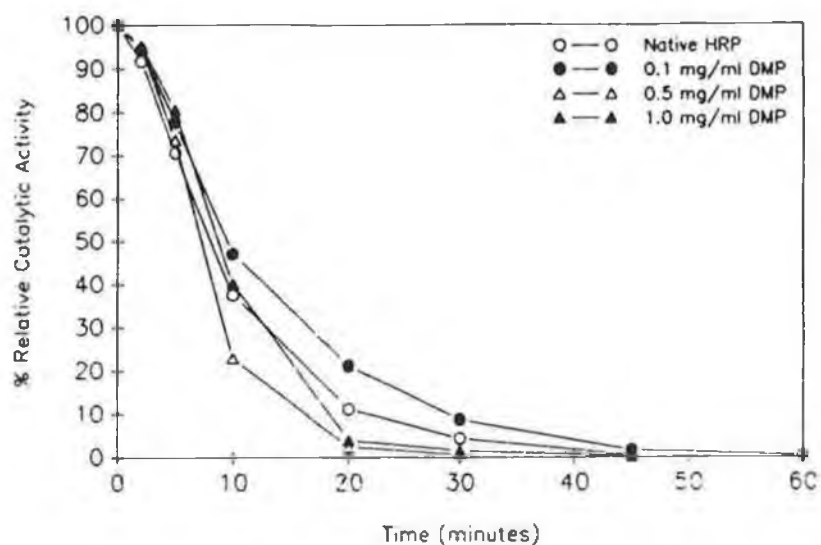Fig. 4.13.   Structure of dimethyl adipimidate.

Fig. 4.11.   Thermoinactivation at 72.5°C of DMP samples that were modified 3
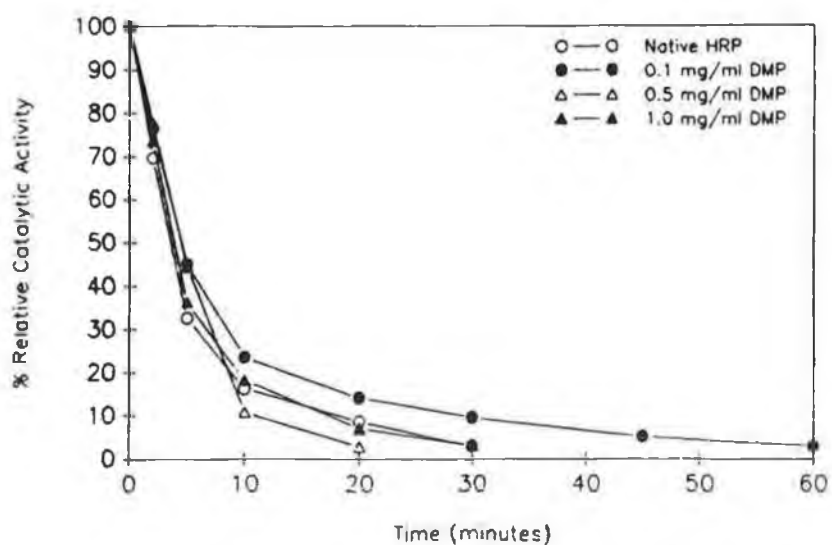days previously.

Fig. 4.12.   Thermoinactivation at 72.5°C of borohydride treated DMP stocks.

Table 4.4.   Table of apparent half-lives of DMP-treated HRP samples.

FIGURE	SAMPLE	$t_{1/2}$ (min)
4.9 (DAY 0)	Native HRP	5
	0.1 mg/ml DMP	4
	0.5 mg/ml DMP	5
	1.0 mg/ml DMP	15
4.10 (DAY 1)	Native HRP	12
	0.1 mg/ml DMP	45
	0.5 mg/ml DMP	18
	1.0 mg/ml DMP	23
4.11 (DAY 3)	Native HRP	8
	0.1 mg/ml DMP	11
	0.5 mg/ml DMP	7
	1.0 mg/ml DMP	9
4.12 (reduced)	Native HRP	5
	0.1 mg/ml DMP	6
	0.5 mg/ml DMP	6
	1.0 mg/ml DMP	5

The length of the molecular crosslink formed by DMA is 9.0Å. Again, the reaction details and optimum conditions cited for DMS in Section 4.1.2.1. apply here. DMA concentrations of 0.1 mg/ml to 1.0 mg/ml were used to modify HRP (1 mg/ml) pH 7.0. For isoenzyme C, higher concentrations of DMA were used, up to 10.0 mg/ml DMA. However, no successful crosslinking was seen for isoenzyme C under any reaction conditions. Successful crosslinking of HRP with DMA was seen at a DMA concentration of 0.5 mg/ml, and also to a lesser extent at 0.1 mg/ml DMA. (See Fig 4.14). Fig. 4.14 depicts the thermal decay at approx. 72.5°C of DMA stocks on the day the modification experiment was performed. Although crosslinking is evident, it is not very pronounced and any effects of enhanced thermal stability have disappeared after 45 minutes. Three days after this experiment was performed, the crosslinking of HRP by DMA has reached its maximum (See Fig 4.15). Very good stability towards heat denaturation is seen for the 0.5 mg/ml DMA sample, which retains 30.9% of its original activity after heating for 60 minutes. For the 0.1 mg/ml DMA sample, the thermal stability seen is somewhat better than that seen for the native enzyme, though the extent is not as pronounced as for the 0.5 mg/ml sample. The 1.0 mg/ml DMA sample shows less thermal resistance than the native enzyme. For all samples, as has been the case for all bis-imidates, no loss in activity occurs due to effect of modification reaction. Fig. 4.16 shows the thermal decay pattern for DMA stocks that were prepared 5 days previously. The crosslinking effect is still present here. Fig. 4.17 shows the thermal decay of DMA stocks that are 11 days old. As can be seen, no enhanced thermostability is present 11 days after modification experiment and all samples decay at about the same rate as native sample. Table 4.5 shows the apparent half-life values for all DMA stocks.

Borohydride treatment of DMA samples was performed as described in Section 2.7.1, i.e. according to the method of Tijssen (1985). Samples that were treated with borohydride had been modified 1 day previously. Four days after this borohydride treatment, the thermoinactivation of the samples was studied (results not shown). The use of borohydride had an adverse effect on the overall activity and thermostability of the DMA samples. Crosslinking that gave rise to enhanced thermostability, as seen in Figs. 4.14, 4.15 and 4.16, no longer seems to be present.
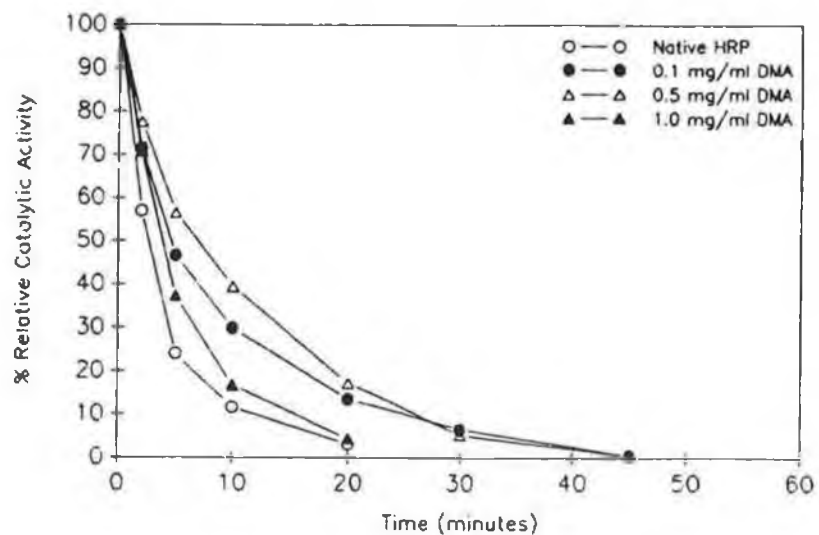
Fig. 4.14.   Thermoinactivation at 72.5°C of DMA treated HRP stocks.
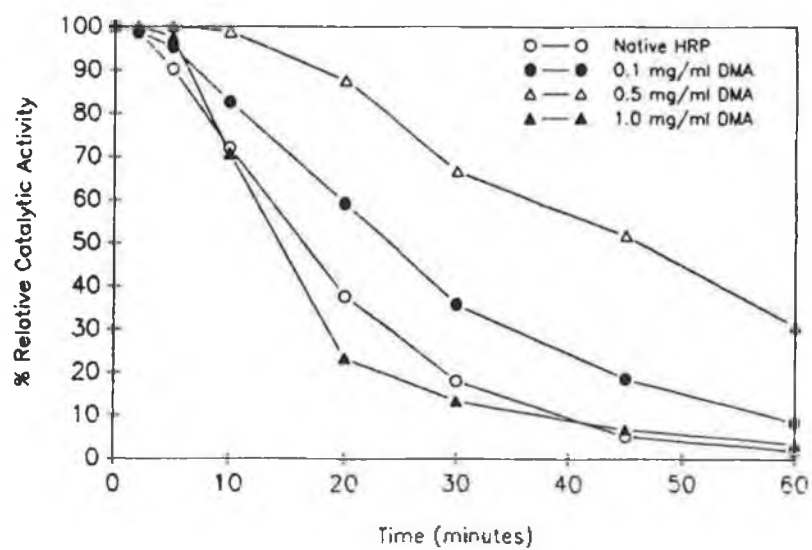

Fig. 4.15.   Thermoinactivation at 72.5°C of 3 day old DMA samples.
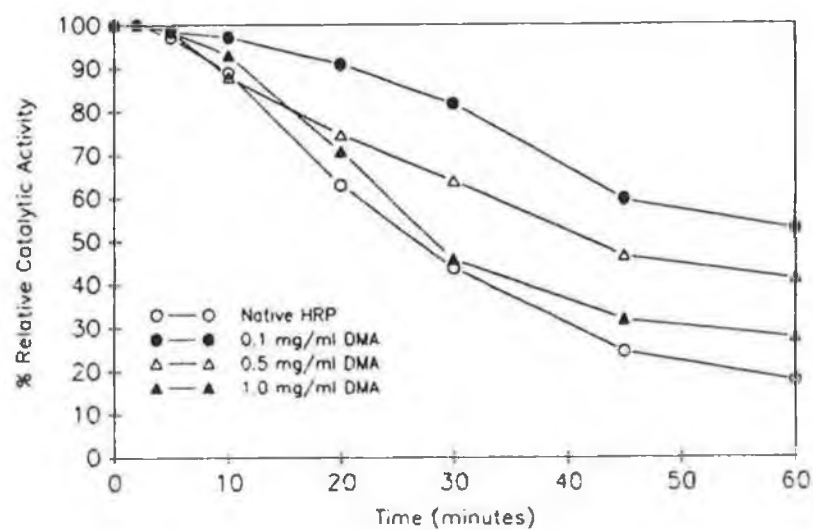
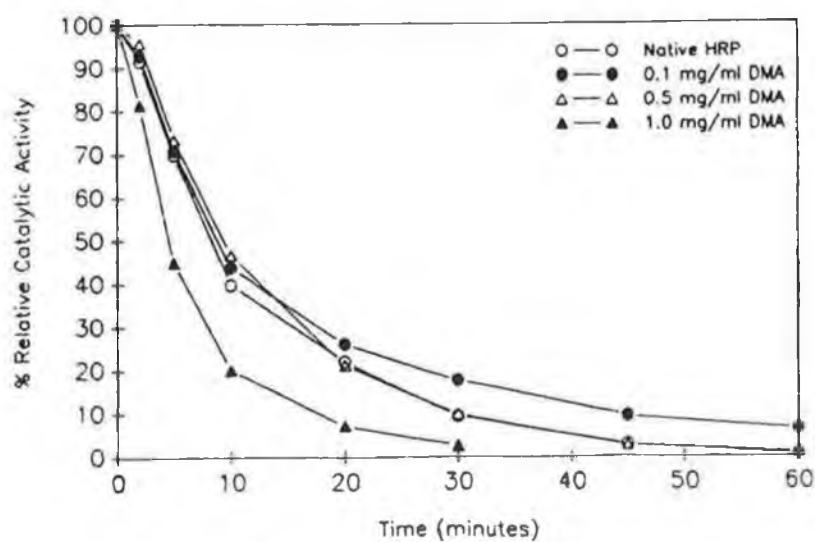Fig. 4.16.   Thermoinactivation at 72.5°C of 5 day old DMA samples.

Fig. 4.17.   Thermoinactivation at 72.5°C of 11 day old DMA stocks.

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Table 4.5.   Table of apparent half-lives for DMA samples.

FIGURE	SAMPLE	$t_{1/2}$ (min)
4.14 (DAY 0)	Native HRP	4
	0.1 mg/ml DMA	6
	0.5 mg/ml DMA	8
	1.0 mg/ml DMA	5
4.15 (DAY 3)	Native HRP	18
	0.1 mg/ml DMA	25
	0.5 mg/ml DMA	48
	1.0 mg/ml DMA	15
4.16 (DAY 5)	Native HRP	28
	0.1 mg/ml DMA	> 60
	0.5 mg/ml DMA	44
	1.0 mg/ml DMA	30
4.17 (DAY 11)	Native HRP	9
	0.1 mg/ml DMA	10
	0.5 mg/ml DMA	11
	1.0 mg/ml DMA	5

Borohydride has not stabilized the crosslinks as expected, but appears to have actually disrupted or removed them. The native enzyme showed the best tolerance of the elevated temperature in this instance. Fig. 4.16 shows the corresponding samples that were not treated with borohydride. So, it is known that crosslinks are still present at this stage. However, borohydride treatment has not resulted in any stabilization of these crosslinks.

Because crosslinks are still present after 5 days (See Fig. 4.16), further characterization of HRP modified with DMA was carried out. Increased resistance to heating at an elevated temperature is only one property of an enzyme that may be altered due to modification. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on DMA crosslinked samples as described in Section 2.4. After the gel was stained with Coomassie blue 4 bands were visible, all of which appeared at the same position on the gel, i.e. at approx. 48,000 (Not shown). The native and modified samples all move to this position on the gel. The fact that all the bands move to similar positions indicates that no intermolecular crosslinks have been formed. No oligomers have been formed between different enzyme molecules. Therefore, there has been no change in molecular size of the modified enzyme. The increased thermal stability seen for DMA samples must therefore be due to intramolecular crosslinks.

DMA samples were also analyzed by high performance liquid chromatography as described in Section 2.11. Any differences in molecular weights of the samples should be discernible here. Chromatograms of absorbance vs. time for each sample reveal peaks that all have similar peak areas. All these peaks have the same retention time, i.e. they are eluted from the column at the same time indicating that they have the same molecular weight. Therefore, no large change in molecular weight of the modified samples has occurred. The formula weight of DMA is 245.1g and the molecular weight of HRP is approx. 40,000 daltons. The increase in molecular weight in modified HRP due to DMA molecules that have formed intramolecular crosslinks is negligible and is too low to show up on HPLC. Overall, the only altered property of DMA modified HRP appears to be enhanced

thermostability.

4.1.3. <u>Summary</u>:

Horseradish peroxidase was modified with three bis-imidates: dimethyl suberimidate, dimethyl pimelimidate and dimethyl adipimidate. The object of these modification experiments was to crosslink HRP. Enhanced thermal stability was taken as an indication that crosslinking had occurred. The reason for enhanced thermostability in a crosslinked sample is due to the prevention of unfolding of the protein backbone. The introduced crosslinks decrease the conformational mobility of the modified enzyme and so the enzyme will unfold less easily under adverse conditions. Therefore, the enzyme is said to be stabilized. The most successful crosslinking was seen with dimethyl adipimidate and to a lesser extent for dimethyl suberimidate. Dimethyl pimelimidate was the least successful bis-imidate used. The main parameters that were found to be important with respect to the final degree of crosslinking were: protein concentration, molar excess or ratio of crosslinking reagent to protein, pH and ionic strength. The use of optimum parameters will ensure that protein-crosslinker interactions are at a maximum during the chemical modification reaction and therefore crosslinking should occur. Although stabilization was seen with respect to increased resistance to heat denaturation, the stabilization effect was usually still not apparent after a 60 minute incubation time. It appears that the degree of stabilization conferred is related to the length of the crosslinker used. The molecular length of dimethyl adipimidate is 9.0Å and this seems to suit HRP. Further characterization of DMA modified HRP by SDS-PAGE and HPLC indicates that no other properties of HRP have been altered by modification. For all bis-imidates, no adverse effect on activity was seen due to modification reaction. Any degree of crosslinking, and hence enhanced thermostability, that was seen for the bis-imidates lasted for only a few days. In some cases, there was enhanced stability one day after modification. There is apparent bond reversal and reformation, i.e., selfstabilization. The lysines on HRP that react initially with incoming modifier may not form bonds that lead to the most stable HRP possible. With time, other lysine-imidate crosslinks may form that may be more durable. The effect is due to kinetic

versus thermodynamic reactions (Mozhaev & Martinek, 1982). An attempt was made to make permanent the crosslinks formed. This was successful only to a small extent in the case of dimethyl suberimidate and not at all for the other imidates. The advantages of using imidates are their high level of solubility in aqueous media, their high degree of specificity for primary amino groups and the fact that they react under mild conditions. The stabilization seen with bis-imidates can be considered successful.

4.2.   Modification with Methylacetimidate:

4.2.1.   Introduction:

Methylacetimidate, a member of the imidate family of reagents, is a protein modification reagent that selects for the ε-amino group in proteins. The charge on the lysine is not altered upon reaction with methylacetimidate (Hunter & Ludwig, 1972). The structure of methylacetimidate is shown in Fig. 4.18.

$$H_3C - C \overset{\underset{\displaystyle \nearrow}{} \overset{+}{N} H_2}{\underset{\displaystyle \searrow OCH_3}{}}$$
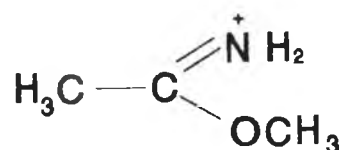
Fig. 4.18.   Structure of methylacetimidate.

The methylacetimidate molecule has one reactive end that binds to lysines and so can be considered to be like one half of a bis-imidate molecule. Fig. 4.19 shows the acetamidination reaction involving methylacetimidate. This acetamidination reaction is a mild modification procedure which results in an acetamidinated lysine that has an arginine-like structure with a similar pK value (Tuengler & Pfleiderer, 1977). The acetamidination of proteins is used as a method for protecting amino groups in proteins (Inman et al, 1983). Although no crosslinking occurs, the lysine molecules will retain their charge and may be protected. Methylacetimidate was used to

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distinguish between stabilizing effects due to crosslinking and any due to single point modification. The degree of stabilization achieved with methylacetimidate will be compared to the stabilization seen with the bis-imidates.
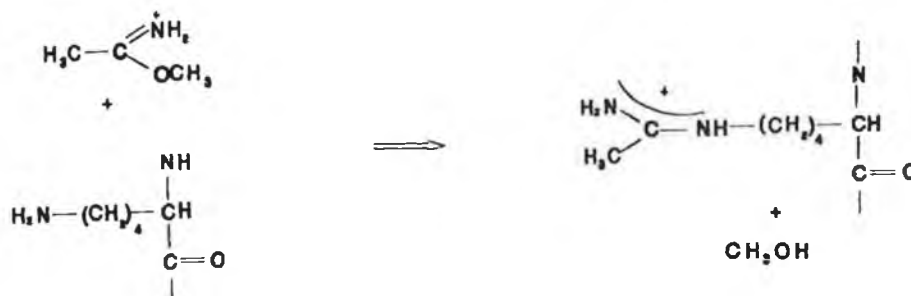

Fig. 4.19.   Methylacetimidate reaction with lysine residue.

4.2.2.   Results and Discussion:

4.2.2.1.   Modification using Methylacetimidate:

Modification of horseradish peroxidase with methylacetimidate (MAI) was performed as described in Section 2.12. The procedures used were based on those that were used for the bis-imidates. Following on from the success of the bis-imidate modification experiments, a set of reaction parameters was available that could be used with methylacetimidate. The range of MAI concentrations used were from 0.1 mg/ml to 5.0 mg/ml. The protein to modifier ratio was 50 : 1 and all the other reaction conditions were similar to those used for the bis-imidates. Some positive results were obtained. For HRP (1 mg/ml) pH 7.0 modified with 0.5 mg/ml and 1.0 mg/ml MAI in phosphate buffer pH 8.0 there was greater thermal stability seen than for native HRP (see Fig. 4.20). After incubation of the 1.0 mg/ml MAI sample for 60 minutes at approx. 72.5°C, 28% of original activity remained. The 0.5 mg/ml MAI sample shows a high level of thermostability for 45 minutes and then it loses nearly all of its activity. Thermoinactivation of methylacetimidate samples was repeated 2 days after initial modification reaction had been performed. Results from this are shown in Fig. 4.21. As can be seen, the 1.0 mg/ml MAI sample is still quite stable and appears to decay at the same rate as two days previously. However, the 0.5 mg/ml MAI sample has lost all of its resistance to heat denaturation present on
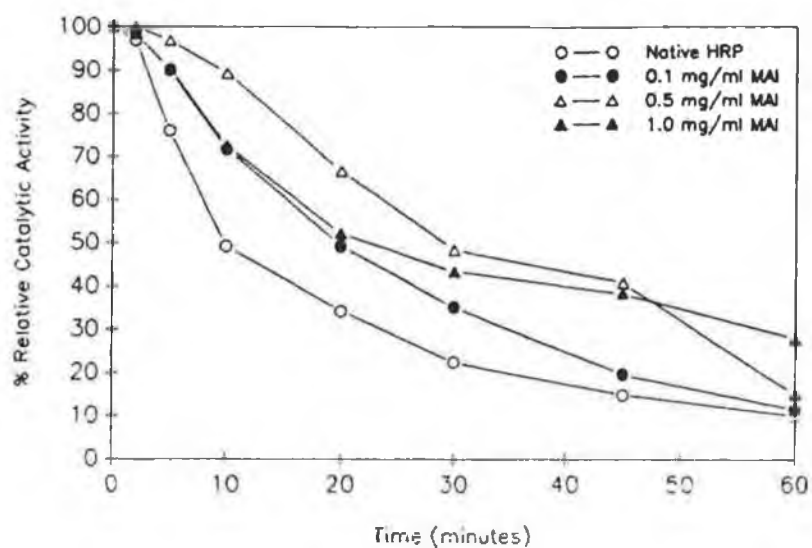
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Fig. 4.20.   Thermoinactivation at 72.5°C of MAI modified samples.
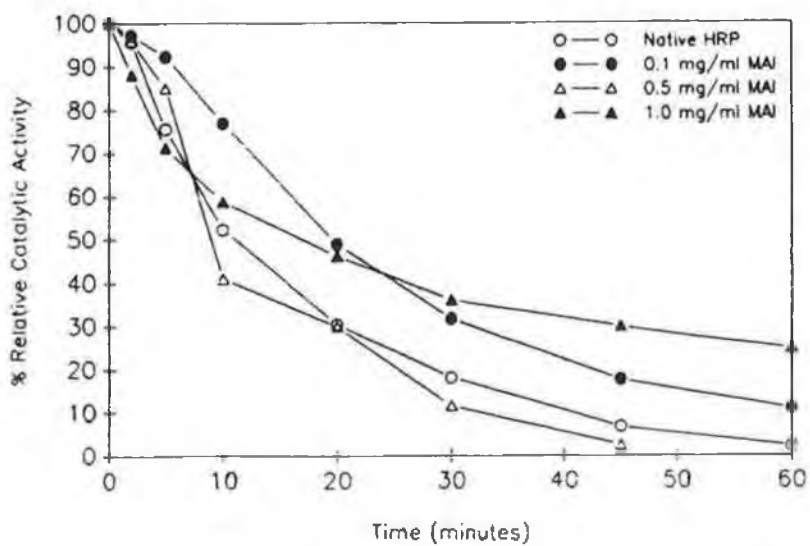

Fig. 4.21.   Thermoinactivation at 72.5°C of 2 day old MAI samples.

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day of experiment and is now less stable than the native enzyme. The 0.1 mg/ml MAI sample still has a moderate degree of resistance to heating. To a certain extent, the thermal stability of HRP shows that acetamidinated HRP is more resistant to denaturation than native HRP. Tuengler & Pfleiderer (1977) give some reasons for the stabilization of acetamidinated proteins towards heat denaturation. The explanations may possibly be that there is an increase in the polar layer around the enzyme or that there are changes in ionic effects due to the change of the ε-amino groups of the lysine residues to arginine-like groups (Tuengler & Pfleiderer, 1977).

Isoenzyme C was modified with methylacetimidate at concentrations ranging from 0.01 mg/ml to 10.0 mg/ml. Both dry methylacetimidate powder and methylacetimidate dissolved in 0.1M phosphate buffer pH 8.7 (Tuengler & Pfleiderer 1977) were used to modify 1.0 mg/ml HRP in phosphate buffer pH 7.0. The pH was measured during the reaction and was found to be fluctuating between these two pH values. For all attempts at MAI modification of isoenzyme C, no enhanced thermostability was seen. None of the modification experiments attempted resulted in stabilized derivatives. This is probably due to the low protein content of isoenzyme C.

Borohydride treatment of methylacetimidate stocks according to the method of Tijssen (1985) as outlined in Section 2.7.1. resulted in a decrease in thermal stability of modified samples. The reason for this may be the fact that no Schiff bases were formed in this acetamidination reaction and so reduction reactions can serve no useful purpose.

As mentioned, methylacetimidate was used as a monofunctional control to distinguish between genuine crosslinking and single point modification. A comparison of the apparent half lives for methylacetimidate and bis-imidate treated stocks is shown in Table 4.6. Comparing the apparent values in this table to those for all bis-imidate samples, it is evident that MAI apparent half-lives are quite high. However, the values are not as high as some DMP and DMA half-life values. It appears that the monofunctional imidoester, MAI, can bring about some increased

Table 4.6.   Table of apparent half-lives for methylacetimidate samples.

FIGURE	SAMPLE	$t_{1/2}$ (min)
4.20 (DAY 0)	Native HRP	10
	0.1 mg/ml MAI	20
	0.5 mg/ml MAI	30
	1.0 mg/ml MAI	25
4.21 (DAY 2)	Native HRP	11
	0.1 mg/ml MAI	20
	0.5 mg/ml MAI	9
	1.0 mg/ml MAI	18

thermostability. But, the effect seen for the bifunctional imidoesters, that bring about true crosslinking, is far greater.

4.2.3. Summary:

Horseradish peroxidase was modified with methylacetimidate in an attempt to increase the thermostability of the enzyme. Some successful modification was seen at a methylacetimidate concentration of 1.0 mg/ml. This result is interesting as modification by methylacetimidate leading to increased thermostability is due to amino group protection, and is not due to crosslinking as is the case for the bis-imidates. The modification of HRP at 1 mg/ml methylacetimidate is still present after 2 days. Borohydride treatment of methylacetimidate stocks led to a loss in activity. As the methylacetimidate reaction may not lead to the formation of Schiff bases, reduction apparently has an adverse effect. No successful modification of isoenzyme C was seen.

4.3. Diamine Modification:

4.3.1. Introduction:

A diamine is a bifunctional amine molecule of a particular length. The diamine used in this Section was ethylene diamine (also referred to as 1,2-diaminoethane). This molecule contains 2 -CH_2- groups and its molecular formula is $NH_2.CH_2.CH_2.NH_2$. This diamine, and any other amine, is not capable of forming crosslinks on its own, but it can form amide linkages when used in conjunction with a water soluble carbodiimide (Torchilin et al, 1983, 1979 and 1978). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) can be used to activate carboxyl groups on proteins. Various reagents, such as diacids, as used by Torchilin et al (1983), and diamines can be introduced into the activated protein mixture to form crosslinks between the activated carboxyl groups and amino groups on proteins.

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4.3.2.   Results and Discussion:

4.3.2.1.   Activation of Carboxyl Groups on Horseradish Peroxidase:

The carboxyl groups on horseradish peroxidase were activated using the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as described in Section 2.13. Carbodiimides are bivalent coupling reagents with the general formula R-N=C=N-R' (Jennes & Stumpf, 1983). The R and R' groups may be aliphatic or aromatic, like or unlike. The nature of these R groups determines the solubility of the carbodiimide (Tijssen, 1985). Carbodiimides can react with several functional groups, such as carboxyl and amino groups, under mild conditions of neutral pH. For the conjugation of thiols, phenols or alcohols more drastic conditions are needed, i.e., acidic pH values. Use of EDC allows the formation of an amide bond between an amine and a carboxylic acid group. Several sets of reactions occur during the coupling of EDC to a functional group. The functional groups in this instance were the carboxyl groups of HRP. The amine used in this case, ethylene diamine, was introduced to the reaction mixture after activation of HRP with EDC.

The structure of EDC is shown if Fig. 4.22. A pH of approx. 7.0 is used to activate carboxyl groups on HRP. At this pH, EDC may also react with amino groups (Torchilin et al, 1983 and 1978).

$$CH_3\!\!-\!\!CH_2\!\!-\!\!N\!\!=\!\!C\!\!=\!\!N\!\!-\!\!(CH_2)_3\!\!-\!\!\overset{\displaystyle Cl^-}{\underset{\displaystyle CH_3}{N^+}}\!\!-\!\!CH_3$$
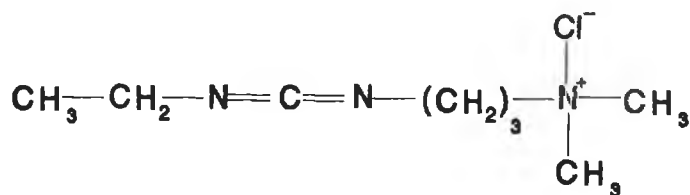
Fig. 4.22.   Structure of EDC.

The reaction of EDC with carboxyl groups is shown in Fig.4.23. Concentration range of EDC used was 1mM to 1M at a 1 : 10 ratio, i.e. 1 part EDC to 10 parts HRP.

Thermoinactivation of HRP samples that had been treated with various concentrations of EDC at pH 4.75 or pH 7.0 showed no enhanced thermostability towards heat denaturation when incubated at 72.5°C for 60 minutes. These results can be taken as monofunctional controls. No modification of HRP leading to enhanced thermostability is evident due to the action of EDC alone. In the absence of an amine, the activated carboxyl groups are usually hydrolysed to regenerate free carboxyl groups (Imoto & Yamada, 1989). Alternatively, activated carboxyl groups in the absence of an amine may react with amino groups on the same or other protein molecules to give either intramolecular crosslinks or intermolecular crosslinked polymers.

4.3.2.2.   Diamine Modification:

Modification of carbodiimide-activated horseradish peroxidase with ethylene diamine was performed as described in Section 2.14. Ethylene diamine, or 1,2-diaminoethane, will react in an amidination reaction with activated carboxyl groups on HRP. Solutions of ethylene diamine were prepared in distilled water and the pH of the solution was approx. 7.0. EDC was prepared in 0.1M phosphate buffer pH 7.0 as in Section 2.13. It is important to keep the reaction mixture at neutral pH (Illum & Jones, 1985). At too low a pH the amine is protonated and does not readily react. At a higher pH the carbodiimide may decompose. The ethylene diamine (EDA) serves as a nucleophile to carbodiimide-activated HRP. Amide bonds are formed between an activated carboxyl group and an amino group of HRP via the introduced amine. Fig. 4.23 shows the amidination reaction of activated carboxyl groups with an added amine in amide bond/linkage formation.
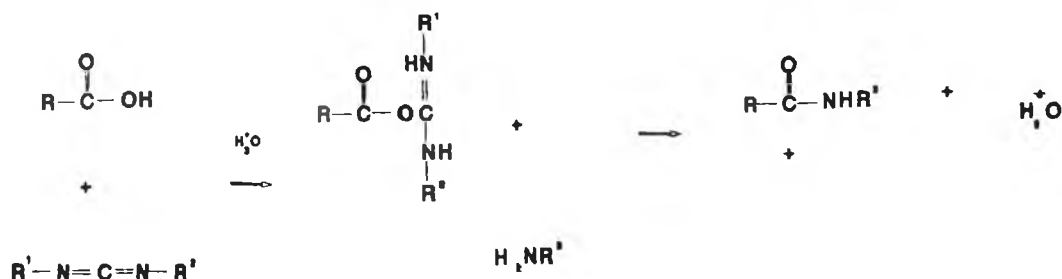

Fig. 4.23.   Amide bond formation.

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Concentrations of ethylene diamine used ranged from 1mM to 1M. These correspond to EDC concentrations used in Section 2.13. As in the case of EDC, EDA was used at 10% final volume. A sample of HRP activated with a certain concentration of EDC was treated with the same concentration of EDA. No adverse effects were seen on HRP activity at any of the concentrations or reaction conditions used. The thermoinactivation of HRP treated with EDC and EDA at varying concentrations is shown in Fig. 4.24. As can be seen, the use of 1mM EDC and 1mM EDA seems to have had a slightly adverse effect on the thermostability of HRP. This may be due to the fact that activated carboxyl groups (if any) may have hydrolysed and this may destabilize HRP. Perfetti et al (1976) found that the modification of papain with EDC lead to its irreversible inactivation. The inactivation was accompanied by a derivatization of catalytically essential thiols of the enzyme and by modification of carboxyl and tyrosyl residues. So, the interference with essential residues of papain led to the EDC-induced inactivation. Similarly, Minotani et al (1979) found that a loss in EDC treated lactate dehydrogenase activity was due to modification of the essential sulfhydryl group in the enzyme, and not to the modification of carboxyl groups. 10mM, 100mM and 1M EDC plus EDA samples have slightly enhanced resistance to thermal denaturation. Perhaps only a few amide linkages are formed at these concentrations. Ethylene diamine was used to interact with α-chymotrypsin that had been activated with carbodiimide by Torchilin et al (1979). Torchilin et al (1983) found that glyceraldehyde-3-phosphate dehydrogenase could be stabilized against deactivation by using different diacids that interact with protein amino groups in different subunits of the enzyme after preliminary activation with water-soluble carbodiimide. The stabilization is due to a decrease in intramolecular (considering the enzyme as a functional oligomer) protein mobility. If crosslinks have been formed in HRP, then the same theory applies. The limited degree of enhanced thermostability seen has been brought about due to a decrease in conformational flexibility of the enzyme. However, it is not known for definite if crosslinking, or one-point modification has occurred. There is a molar excess of approx. 40,000 when using EDC and EDA at 1M. This is an extremely large molar excess, though no adverse effects on activity have been seen. Other amines that could be used include diamino heptane, diamino
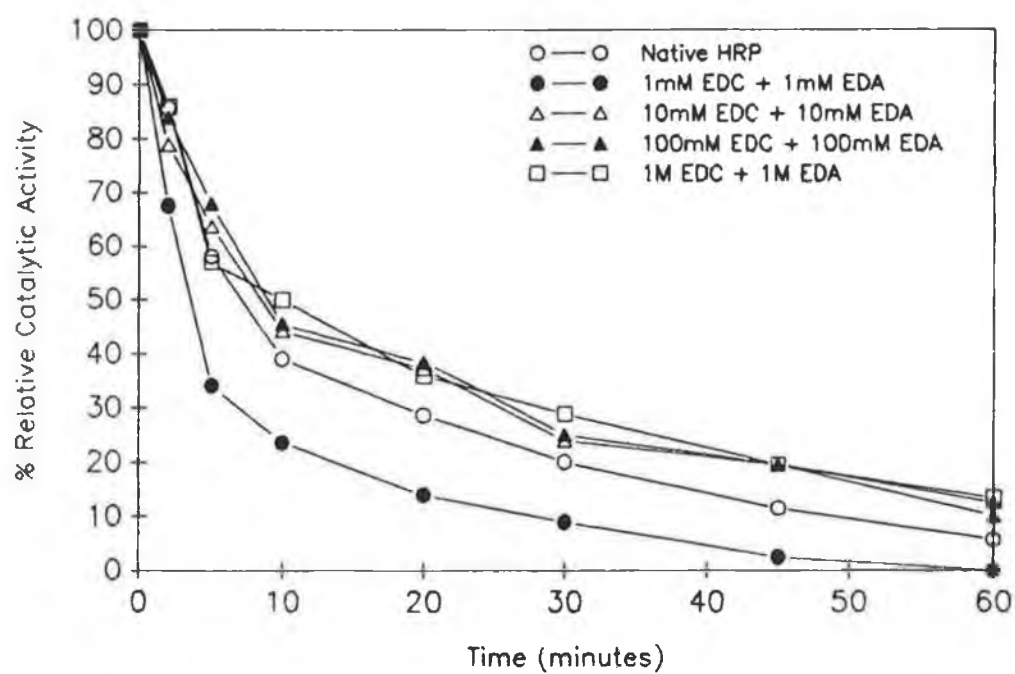
Fig. 4.24. Thermoinactivation at 72.5°C of HRP treated with EDC and EDA at various concentrations.

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octane, diamino decane and diamino dodecane. In fact, appropriate reactive molecules, such as carbodiimide-activated adipic acid and succinic acid (as used by Torchilin et al, 1983) can be used to form amide bonds between activated carboxyl groups and amino groups on proteins. Further work on the use of diamines of different lengths should help to optimize the modification of HRP seen in Fig. 4.25 for 1M EDC and EDA.

The amine used above was ethylene diamine. One of its `NH$_2$ groups has two free electrons and so is able to react. Depending upon the amine employed, the character of the product can be varied considerably. The resulting product may have an ionic character like that of the carboxyl group replaced (anionic), or it may be neutral, or even cationic. The product formed with ethylene diamine is cationic (Means & Feeney, 1971).

The presence, or absence, of activated carboxyl groups and amide linkages in Fig. 4.24 is speculative. The extent of modification of carboxyl groups can be measured if one employs an amine that is detectable by amino acid analysis after hydrolysis of HRP. The extent of the carbodiimide and amine reaction can also be determined by studying the incorporation of a radioactive amine (Imoto & Yamada, 1989, Means & Feeney, 1990). Carbodiimide amidination was first used to modify proteins and has been used in immunochemical studies and in tissue fixation. Uses include RNA-protein crosslinking (Expert-Bezançon & Chiaruttini 1988), preparation of antibody conjugates (Goodfriend et al, 1964), coupling of antibodies to latex microspheres (Illum & Jones, 1985) and, as mentioned previously, in studies on the essential residues in papain. The advantages of using the carbodiimide amidination coupling procedure as a chemical modification technique are that the reactions are simple to perform and proceed under very mild conditions.

4.3.3.   Summary:

Activation of carboxyl groups on horseradish peroxidase with carbodiimide and introduction of ethylene diamine as an amine was performed in order to form amide

bonds between activated carboxyl groups on the enzyme molecule. Some amide bond formation, that led to slightly enhanced thermostability was observed for the 10mM, 100mM and 1M EDC/1M EDA, pH 7.0 samples. Only one amine was studied. Future optimization of this method could be performed using a variety of different amines of different lengths.

4.4.   Crosslinking with N-hydroxysuccinimide Esters:

4.4.1.   Introduction:

Bis-N-hydroxysuccinimide esters are homobifunctional crosslinking reagents, i.e., they have two reactive groups, one at either end of the molecule. A series of esters of succinimide of various chain lengths are available. The N-hydroxysuccinimde (NHS) esters used in this Section were suberic acid (SA) ester of NHS and ethylene glycol succinic acid (EG) ester of NHS. The structures of these two esters are shown in Fig. 4.25(a) and 4.25(b), respectively.
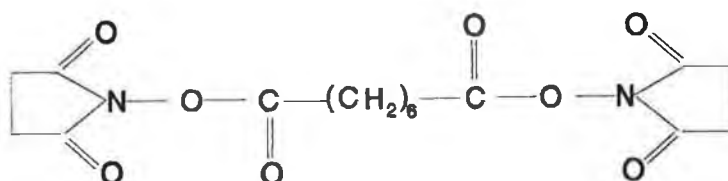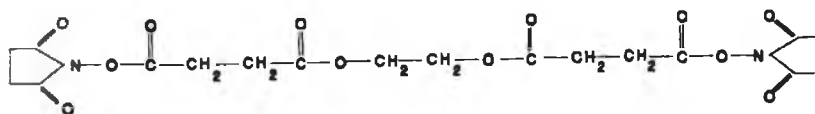

Fig. 4.25(a).   Suberic acid-bis(NHS ester).

Fig. 4.25(b).   Ethylene glycol-bis(succinic acid NHS ester).

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The maximum molecular linkage length of SA-NHS is 11Å (Ji 1983) and that of EG-NHS is 16.1Å. The NHS esters used in this Section are derivatives of suberic acid and ethylene glycol (Han et al, 1984). Both reactive ends of these molecules are amino specific. They react preferentially with primary amino functions such as ε amine groups of lysines or available N-termini. At neutral to alkaline pH, i.e. 6.0 - 9.0, the amino group on a particular ligand will react with an NHS ester to form a stable amide bond, releasing N-hydroxysuccinimide as a by-product. The NHS ester reaction scheme is depicted in Fig. 4.26. The principal product of the reaction with an amine is an amide so the positive charge on the original amino group is lost. The reaction involves the nucleophilic attack of an amine on the ester carbonyl of an N-hydroxysuccinimide ester to form an amide, with release of the N-hydroxy succinimide (Ji 1983).
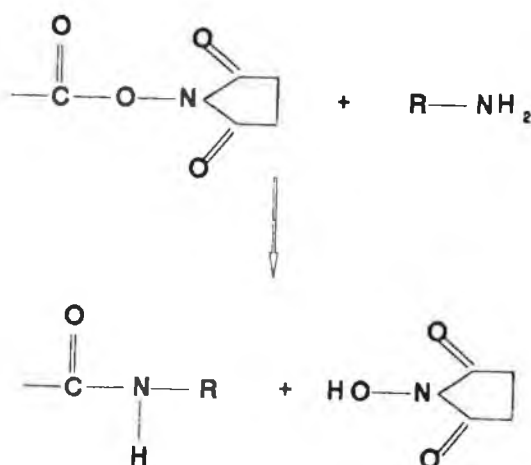

Fig. 4.26.   NHS ester reaction scheme.

The reaction proceeds most efficiently at pH 7.0 - 8.0. The more alkaline the pH of the reaction mixture, the faster the rate of reaction. The NHS ester reaction in aqueous solution consists of two competing reactions. One is the reaction with the primary amine and the other is hydrolysis of the NHS ester. Hydrolysis inactivates the NHS ester and so decreases efficiency of the crosslinking reaction. Hydrolysis is favoured in dilute protein solutions and acylation is favoured in more concentrated protein solutions. The rapid reaction ensures that most accessible protein amino groups are attacked within 10 - 20 minutes. A 10 fold molar excess of an NHS ester is usually sufficient to acylate amino groups. NHS esters react over a wide range

of temperatures, i.e., 4 - 25°C. The reaction buffer should be free of extraneous amines such as Tris or glycine.

The major advantages of the NHS esters that have resulted in their becoming extremely popular are their mild and speedy reaction conditions and their high chemical reactivity and specificity. One drawback is the fact that NHS esters are not readily soluble in aqueous buffers (Ji, 1983). The NHS esters must be dissolved initially in a minimal amount of an organic solvent, such as dimethyl sulphoxide, dimethyl formamide or acetone. The NHS ester in organic solvent forms an emulsion which will allow the reaction to occur.

Crosslinking was performed using the NHS esters mentioned and all the above details were taken into consideration. NHS derivatives of HRP that were successfully stabilized were characterized to determine if any properties of HRP had been altered.

4.4.2. <u>Results and Discussion:</u>

4.4.2.1. <u>N-hydroxysuccinimide Modification of Horseradish Peroxidase:</u>

Crosslinking of horseradish peroxidase with suberic acid N-hydroxysuccinimide ester (SA-NHS) and ethylene glycol bis(succinic acid N-hydroxysuccinimide ester) (EG-NHS) was performed as described in Section 2.15.1. N-hydroxysuccinimide was used as a monofunctional control at similar concentrations and conditions. 1.0 mg/ml HRP pH 8.0 was modified with either 1mg SA-NHS or 1 mg EG-NHS dissolved in 5% v/v dimethyl sulphoxide. Assay for initial recoveries after the crosslinking reaction yielded 100% activity for all samples. Thermoinactivation of HRP-NHS samples and controls is shown in Fig. 4.27. As can be seen, very successful stabilization has occurred. The stability towards heat denaturation of NHS modified samples is remarkable. SA-NHS loses 6.4% and EG-NHS loses 22.2% of original enzyme activity after 60 minutes incubation at 72.5°C. Table 4.7 shows the apparent half-life values for NHS-modified HRP samples. The remarkable

thermostability seen is evident as the half lives for both NHS ester samples are greater than 60 minutes. The monofunctional NHS ester did not bring about any significant alteration in thermal decay pattern. It is probable that the NHS ester bound to HRP in a one-point modification, though this one-point modification did not lead to enhanced thermostability. The experiment that gave the results seen in Fig. 4.27 was repeated several times to verify the positive results. In all cases similar results were obtained. In some cases the EG-NHS derivatives appeared more thermostable than the SA-NHS-HRP, and vice versa. The most thermostable HRP derivatives from all of the experiments were stored at 4°C for use in derivative characterization. At various intervals over a two week period the thermal stability of the NHS modified samples was determined. After all the time intervals tested, thermostability of modified derivatives was still evident. Fig. 4.28 depicts the thermal decay of NHS ester-HRP samples that were modified six days previously. The thermostability of stocks was assayed two weeks after they were prepared. Enhanced thermostability, and therefore crosslinking, was still present (See Fig. 4.29). Long term stability, such as is evident after two weeks for NHS ester-treated samples, is desirable if stabilized derivatives are to be used in industrial and other applications. True crosslinking has occurred since the monofunctional control had no effect on thermostability.

The results for NHS modification experiments indicated that the high degree of stabilization is due to crosslinking. Because of this, characterization of NHS derivatives was performed by a number of methods as described in Section 2.15.2 - 2.15.7. The enhanced thermostability that is seen in NHS treated HRP samples is presumably due to decreased conformational mobility of the enzyme backbone that has been brought about due to intramolecular crosslinks. In the NHS crosslinking reaction the positive charge of the original amino group is lost. This charge alteration may also be a contributing factor in the enhanced thermostability effect. Generally, NHS esters are used in crosslinking and structural studies. N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) is a heterobifunctional NHS ester that is frequently used in preparation of enzyme-antibody conjugates for enzyme linked immunosorbent assays (Tijssen, 1985). NHS esters have also been used in
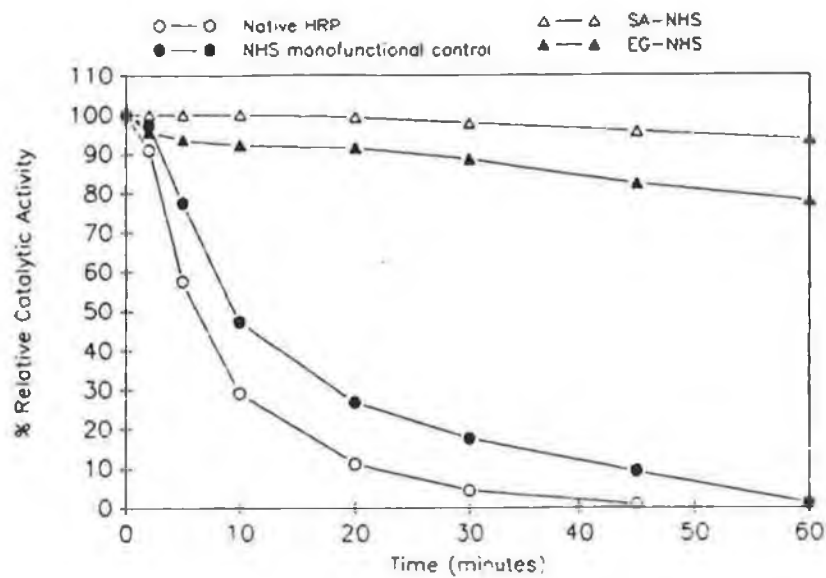
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Fig. 4.27.   Thermoinactivation at 72.5°C of NHS ester-HRP samples.
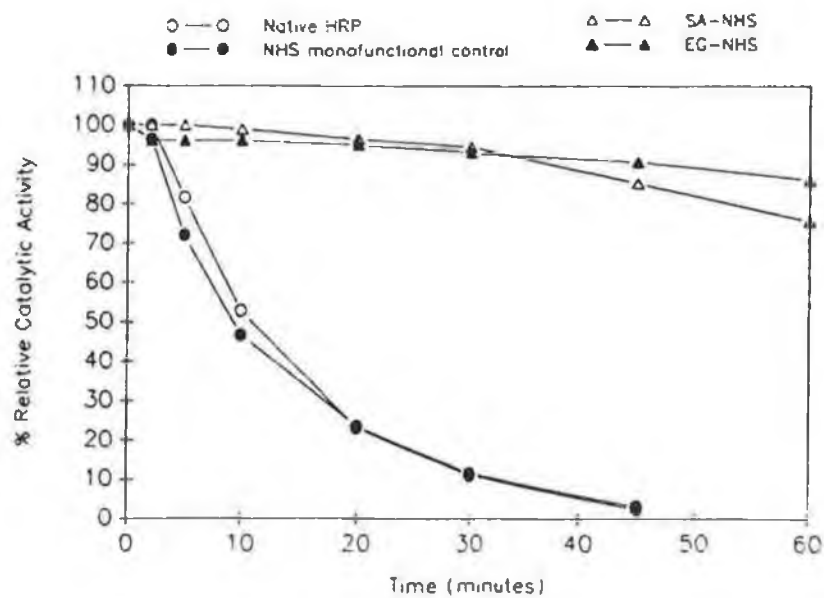

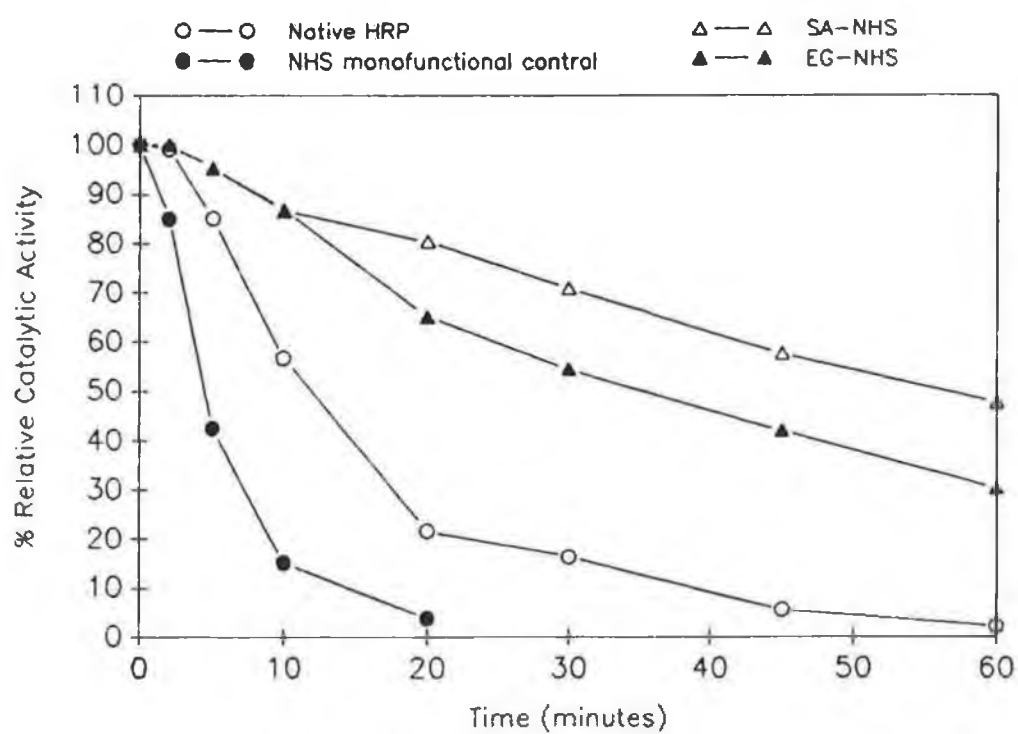Fig. 4.28.   Thermoinactivation at 72.5°C of 6 day old NHS ester-HRP samples.

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Fig. 4.29.   Thermoinactivation at 72.5°C of 11 day old NHS ester-HRP samples.

Table 4.7.   Table of apparent half-lives for NHS ester-modified HRP samples.

FIGURE	SAMPLE	$t_{1/2}$ (min)
4.27 (DAY 0)	Native HRP Monofn. NHS SA-NHS-HRP EG-NHS-HRP	7 10 > 60 > 60
4.28 (DAY 6)	Native HRP Monofn. NHS SA-NHS-HRP EG-NHS-HRP	11 10 > 60 > 60
4.29 (DAY 14)	Native HRP Monofn. NHS SA-NHS-HRP EG-NHS-HRP	13 6 59 37

the study of membrane receptors (Massague et al, 1981, Herberg & Iyengar, 1985).

4.4.2.2. Characterization of NHS Derivatives:

After the initial thermal characteristics of HRP modified with NHS esters were established, several other methods of characterization of the NHS derivatives were performed. These were performed in an attempt to ascertain if intramolecular or intermolecular crosslinks had been formed and to determine if any other properties (besides enhanced thermostability) of HRP had been altered during modification. A crosslinked or modified protein may be more conformationally stable than the native one and so should exhibit enhanced thermostability. Studies on thermostability are quick and easy to perform and only basic laboratory instruments are required. Other relatively simple methods used to study characteristics of native and modified enzymes include the traditional determinations of the kinetic parameters, Km and Vmax and the determination of pH optima. More advanced methods used to study protein conformation and stability include estimations of certain constants such as Gibbs energy, e.g., $\Delta G(H_2O)$ and $\Delta G(25°C)$, from urea or guanidine hydrochloride denaturation curves and estimates from thermal denaturation curves at 25°C, respectively (Pace, 1990). These values, determined using various equations (See Pace et al, 1989), can be used to estimate differences in stability between two proteins differing slightly in structure. HRP modified in a particular way is considered to be different to native HRP, as modified HRP may have an alteration in structure or side chain groups, etc. Pace et al (1989) give details on various techniques that can be used to follow unfolding that occurs in urea or guanidine hydrochloride or to follow thermal decay curves. These techniques include UV difference spectroscopy, fluorescence, circular dichroism, biological activity measurements, optical rotatory dispersion (ORD), nuclear magnetic resonance (NMR), viscosity and other hydrodynamic methods. Determination of thermodynamic constants using one of the techniques mentioned here can be time consuming as a lot of preparative work must be done initially and also expensive equipment and technical expertise may be required. The use of spectroscopy, for the analysis of both absorption and fluorescent spectra, and for the study of the effects

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of denaturing agents (such as urea and guanidine chloride) on protein conformation, is useful. Spectroscopy can be used for the simple and straightforward study of modified proteins. Many other laboratory techniques can be used to give information regarding the conformation and possibly the stability of a modified enzyme. The techniques cited in research papers dealing with protein modification include SDS-PAGE, chromatography and sedimentation velocity experiments. Hunter & Ludwig (1972) cite methods for the determination of the extent of modification with imidoesters. These include ^{14}C-labeled imidoester incorporation studied by radioactivity measurements and reaction of unmodified and modified samples with trinitrobenzene sulphonate and dinitroflurobenzene. Torchilin et al (1979) investigated the effect of intramolecular crosslinks and other modifications on enzyme stability and found that the modifications make the enzyme more stable against a wide range of denaturing actions such as temperature, high salt concentrations and denaturing agents. Any technique that assesses the inherent stability of an enzyme or that studies other biochemical properties of that enzyme can validly be used in studies to compare native and modified enzymes.

High performance liquid chromatography was performed on NHS ester-modified samples as described in Section 2.15.2. The retention times for various eluted peaks are shown in Table 4.8. For NHS modified enzyme samples, two peaks were seen. One corresponds to HRP, i.e. the peak at 18 minutes, and the other corresponds to the NHS ester that elutes at 24 minutes. See Fig. 4.30 (a - e) for HPLC chromatograms. When HRP is run alone, the enzyme is eluted at approx. 18 minutes and a solution of NHS ester alone, with no enzyme present, is eluted at approx. 24 minutes. If there were intermolecular crosslinks present and if there had been any large change in molecular weight, a change in appearance and retention times of the peaks would be evident, as apparent molecular weights shift to higher values. Therefore, enhanced thermostability of NHS ester samples must be due to the presence of intramolecular crosslinks and/or charge alteration, and not due to intermolecular crosslinks.

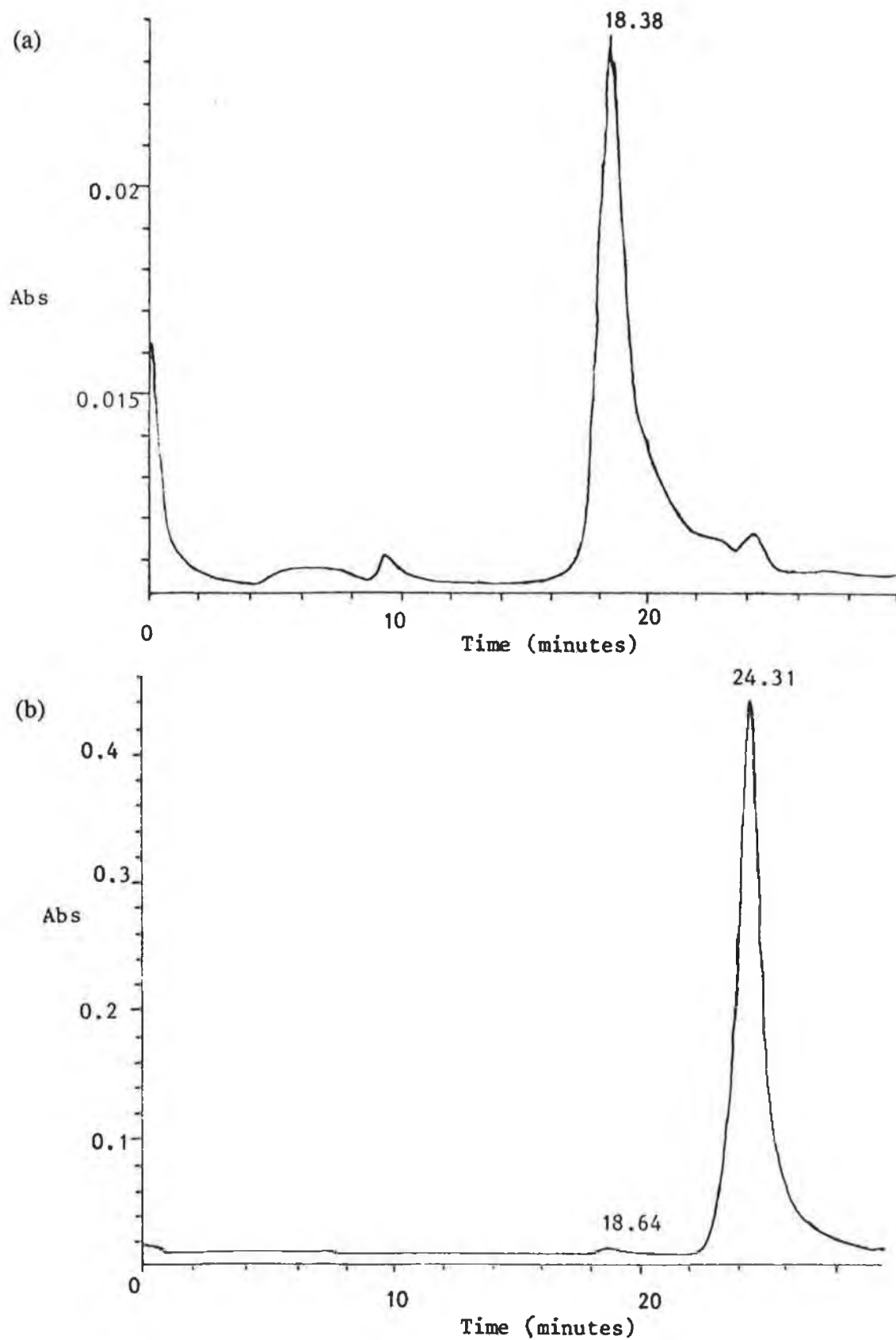Spectral methods can be used to determine and characterize protein conformational

Fig. 4.30 (a) and (b). HPLC chromatograms (absorbance vs. time) of native HRP (a) and monofunctional-NHS HRP (b).
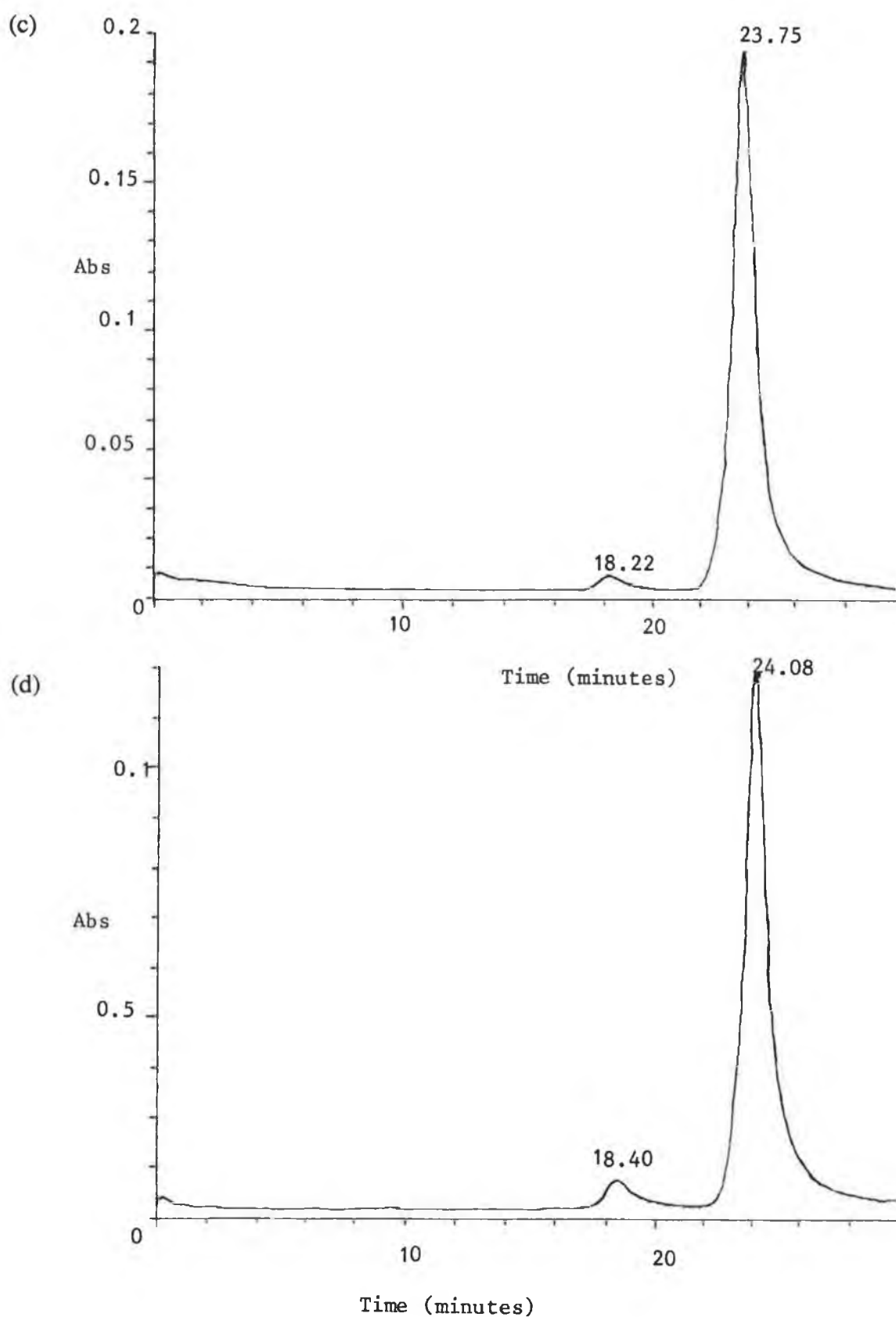
Fig. 4.30 (c) and (d). HPLC chromatograms (absorbance vs. time) of SA-NHS (c) and EG-NHS (d) HRP samples.
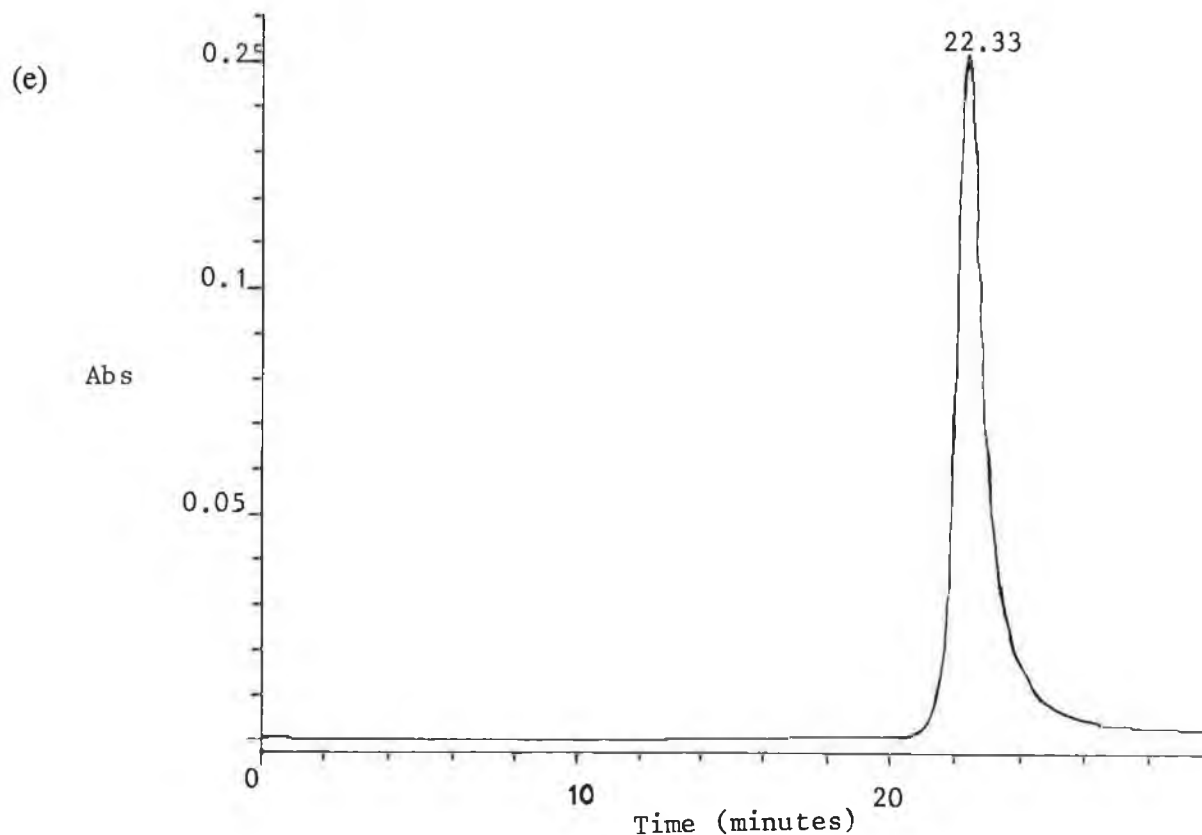
(e)

Fig. 4.30 (e). HPLC chromatogram (absorbance vs. time) of EG-NHS (with no HRP present).

Table 4.8.   Table of retention times for NHS ester-HRP samples run on HPLC.

| SAMPLE	RETENTION TIME * (min) PEAK 1	RETENTION TIME * (min) PEAK 2
Native HRP	18.38	---
Monofunctional NHS	18.64	24.31
SA-NHS-HRP	18.396	24.08
EG-NHS-HRP	18.22	23.75
EG (no enzyme)	----	22.33

* --- indicates that no peak was present.

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changes (Schmid, 1989). Spectral methods are sensitive and only small amounts of protein samples are needed. Samples can be recovered after use and so spectral methods are non-destructive. The u.v./visible absorbance spectrum from 280nm to 540nm of native and modified samples was monitored using a recording spectrophotometer, as described in Section 2.15.3. Spectra are shown in Fig. 4.31 (a - e). A peak is present at approx. 260nm in NHS ester samples that is not present in native sample. This may be due to absorbance of the NHS ester alone. The spectrum for EG-NHS, with no enzyme present, shows a peak at approx. 260nm also. The characteristic absorption spectrum of native HRP shows a major Soret band at 403nm (Ryu & Dordick, 1992). Changes in the microenvironment of the haem present in the active site of HRP can shift the intensity of the Soret band. The peak at 403nm is present in the spectrum for native HRP (see Fig. 4.31(a)). For modified samples, this peak at 403nm seems to have slightly decreased. See Table 4.9 that gives values for the intensity of the peak at approx. 403nm. This may be due to changes in HRP conformation brought about due to NHS ester modification. Overall, there is only a slight difference in absorbance spectra of native and NHS treated HRP.

Horseradish peroxidase has a broad pH range within which it can function, i.e., pH 4.0 - 8.0. NHS ester modification may have altered this, so a study of the pH optimum of modified HRP was performed. A pH profile of native and NHS ester modified HRP was constructed as described in Section 2.15.4. The pH range tested was from pH 5.0 -8.5. A flat profile was observed for all samples, with slightly higher activity seen at higher pH values (pH profile not shown). No great change in the pH profile of NHS crosslinked HRP samples relative to that for native HRP is observed. For some modified enzymes there is a change in pH profile. Sheehan et al (1990) found that creatine kinase modified with DMS had a broader pH profile than that of native creatine kinase. An increase in pH range, together with increased thermostability of a modified enzyme is advantageous, especially if a modified enzyme is to be used in a technical or industrial application.

Fluorescence can be used to investigate conformational changes in proteins (Schmid,
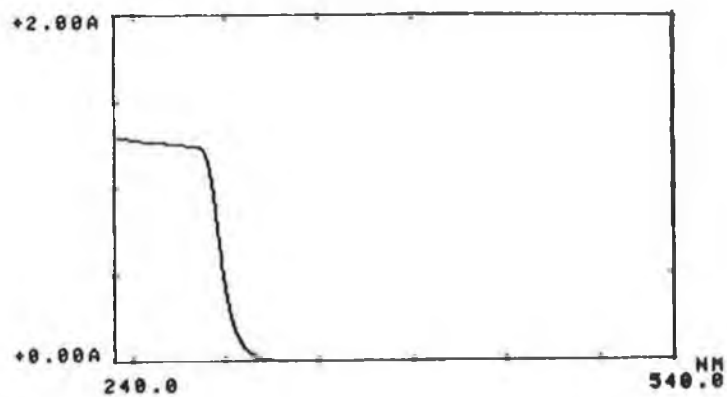
(a)
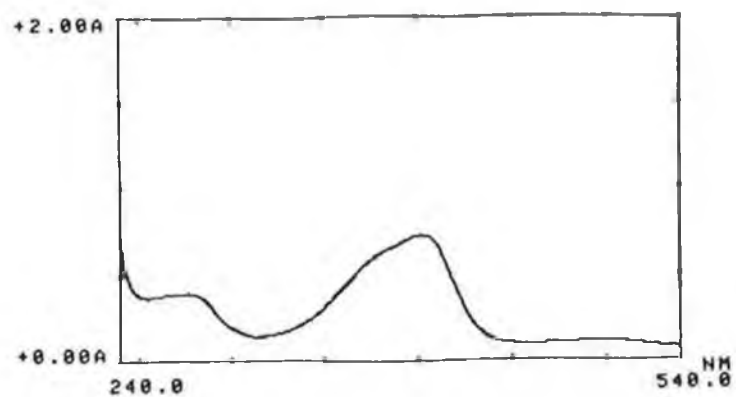
(b)
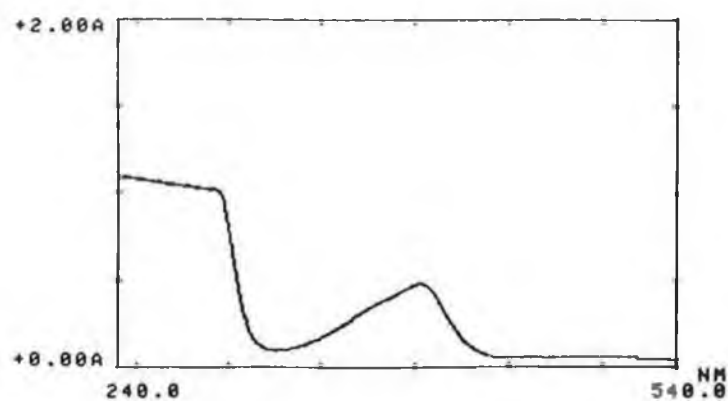
(c)

Fig. 4.31.(a), (b) and (c).   U.V./Visible absorbance spectra (absorbance vs.
wavelength) of NHS ester-HRP (a), monofunctional
NHS-HRP (b) and EG-NHS (no enzyme present) (c)
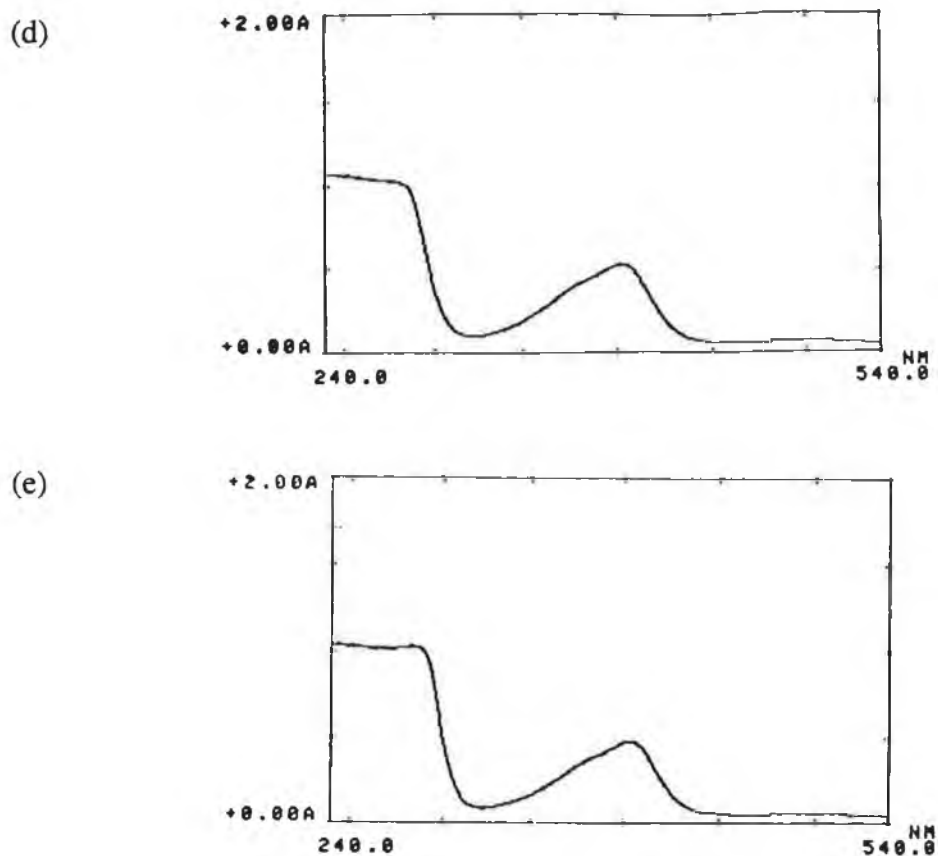
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(d)

(e)

Fig. 4.31. (d) and (e).   U.V./Visible absorbance spectra (absorbance vs. wavelength) for SA-NHS-HRP (d) and EG-NHS-HRP (e).

Table 4.9.   Table of intensity at 403nm of NHS ester-HRP samples.

SAMPLE	ABSORBANCE (403nm)
Native HRP	0.727
Monofn. NHS	0.489
SA-NHS-HRP	0.532
EG-NHS-HRP	0.475

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1989). An excitation wavelength of 280nm was used to study the fluorescent emission spectra of native and NHS modified HRP, as described in Section 2.15.5. Fluorescent emission spectrum of HRP and NHS-treated samples are shown in Fig. 4.32 (a - e). The maximum fluorescent intensity observed in this case for native HRP was approx. 150 at the excitation wavelength and other conditions used in this particular case. (Intensity is measured on a scale of 0 - 1,000). For NHS-treated samples a different emission spectra is seen in which two peaks are visible, the largest having an intensity of approx. 30. For NHS ester alone, without the presence of HRP, the emission spectrum has a peak with a low intensity of 40. The spectra for SA-NHS and EG-NHS are almost identical to each other and to the spectrum for NHS monofunctional control. A problem that was encountered during fluorescent studies was the fact that phosphate buffer used in NHS experiments also fluoresced (spectrum not shown). The intensity of the phosphate buffer peak is approx. 70. The spectrum for phosphate buffer is similar to that for native HRP. Differences seen between the two spectra are: the peak for HRP has higher intensity, has an extra shoulder and is shifted slightly to the right. Overall, the spectra for NHS ester samples are lower than those of control. This may be due to crosslinking/modification. A protein that is more tightly coiled (possibly due to crosslinking) may not fluoresce as strongly as an enzyme in another conformation, as residues that fluoresce may be masked. However, because phosphate buffer also fluoresces, no conclusion can be drawn from these results. The fluorescence of proteins originates from phenylalanine, tyrosine and tryptophan residues. In proteins that contain all three aromatic amino acids, fluorescence is usually dominated by the contribution of tryptophan residues. Schmid (1989) recommends an excitation wavelength of around 280nm if maximum intensity and an excitation of tyrosine and tryptophan are desired. Phenylalanine fluorescence is negligible in a native protein.

SDS-polyacrylamide gel electrophoresis was performed as described in Section 2.15.6. The stained gel showed bands in similar positions for all of the samples. This indicates that no intermolecular crosslinks, or oligomers, had been formed.
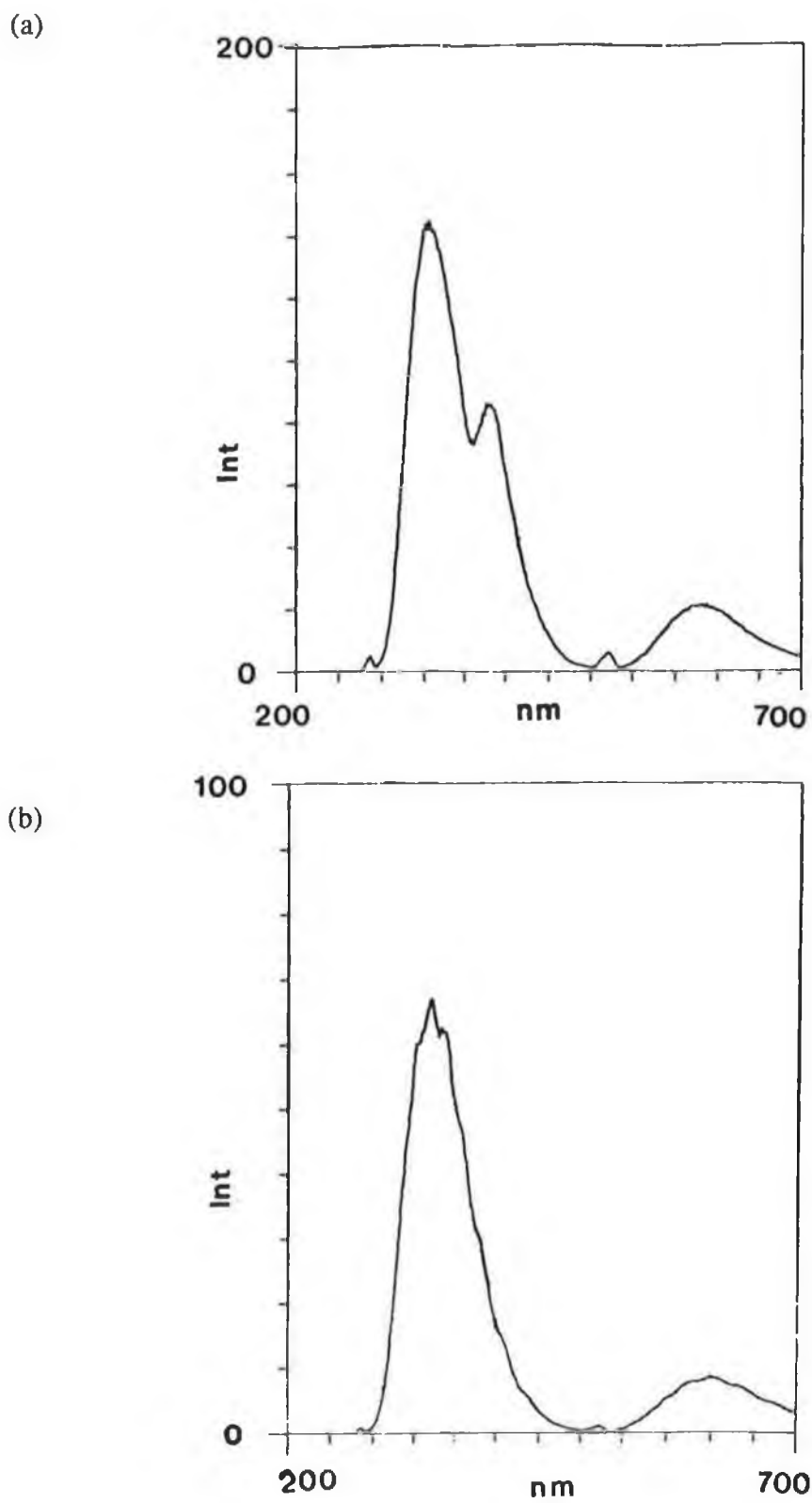
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(a)

(b)

Fig. 4.32. (a and b).   Fluorescent emission spectra (intensity vs. wavelength) of native HRP (a) and phosphate buffer (b).
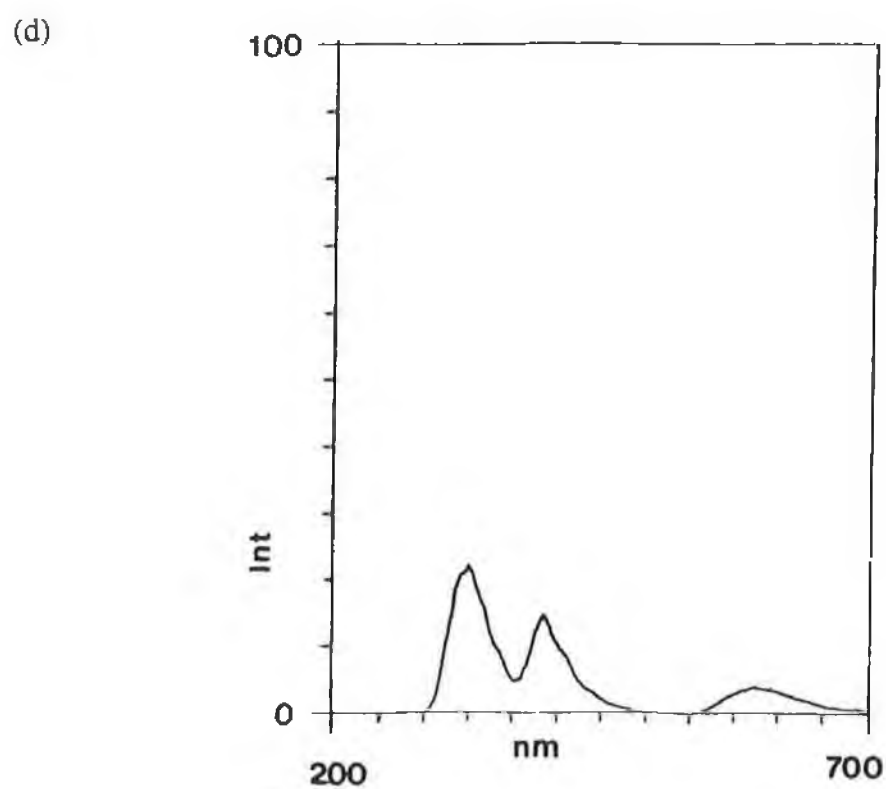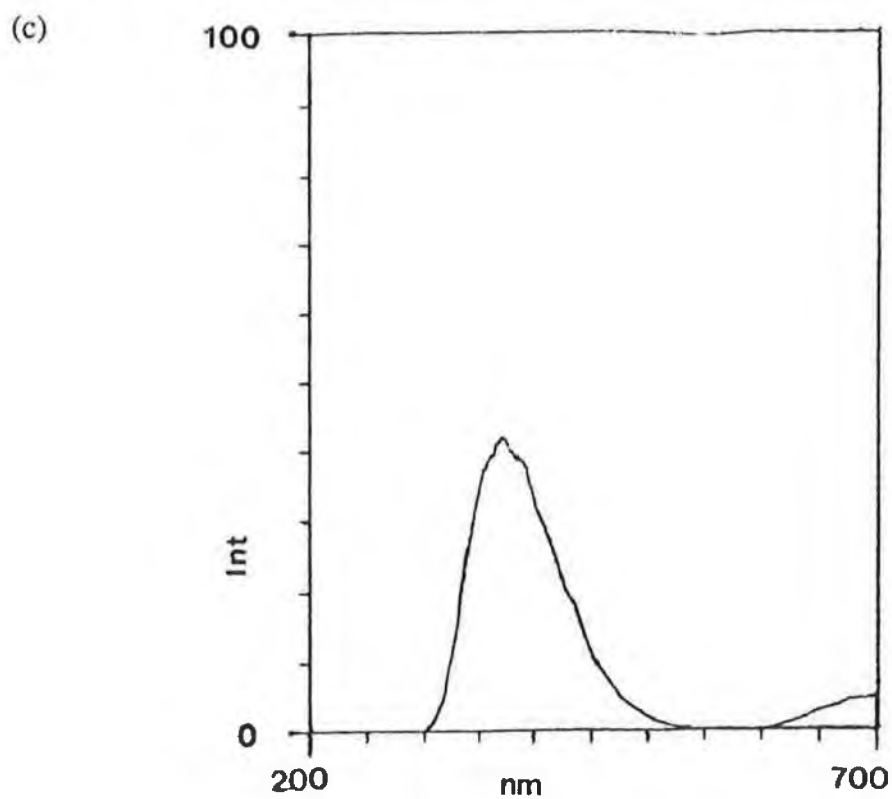
132

Fig. 4.32. (c and d).   Fluorescent emission spectra (intensity vs. wavelength)
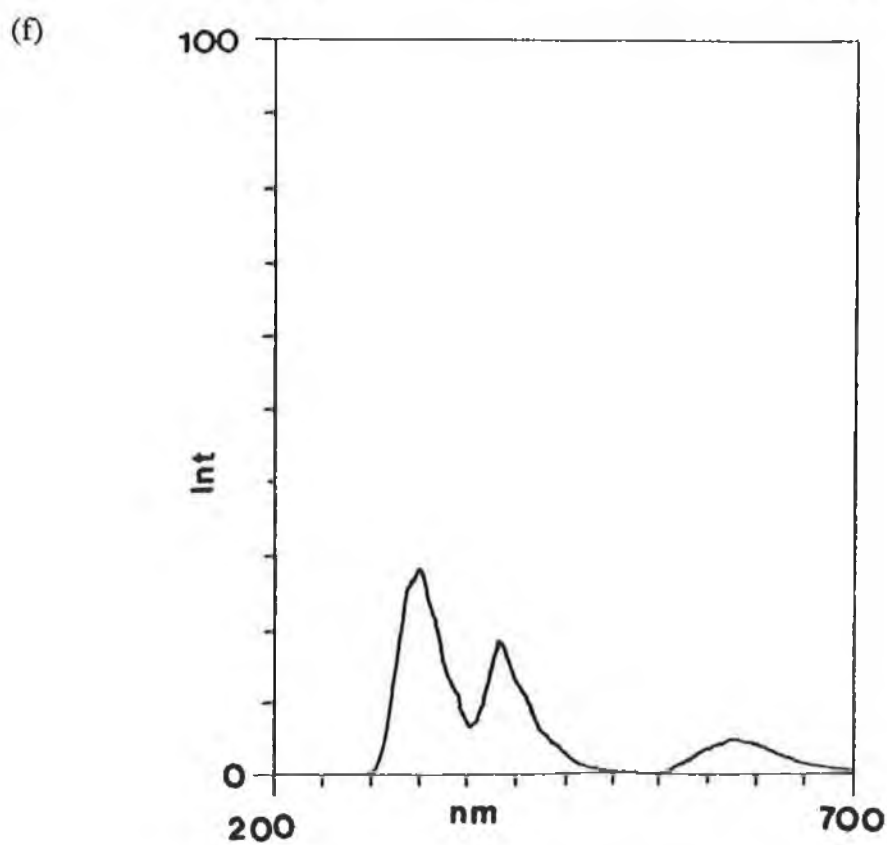of EG-NHS (no HRP) (c) and monofn. NHS-HRP (d).
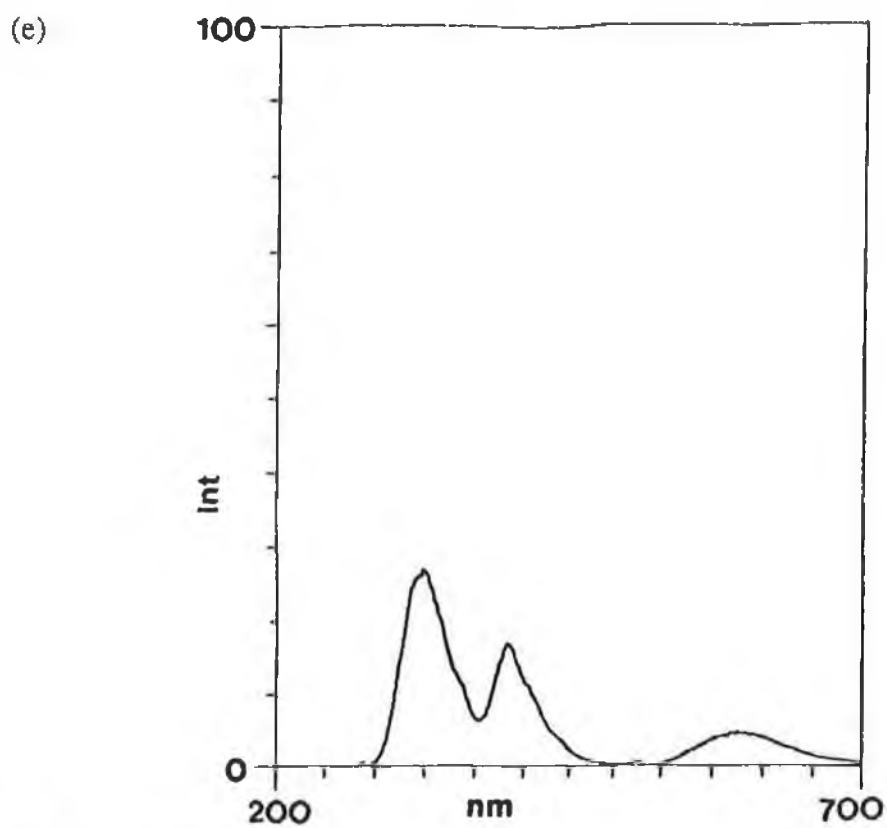
133

(e)

(f)

Fig. 4.32 (e and f).   Fluorescent emission spectra (intensity vs. wavelength) of SA-
NHS-HRP (e) and Eg-NHS-HRP (f).

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A modified enzyme may be more resistant to the effects of a denaturing agent than a native enzyme. The effect of guanidine hydrochloride, a denaturing agent, on native vs. modified HRP was investigated as described in Section 2.15.7. Guanidine hydrochloride (GnCl) is typically used at a concentration of 6M in denaturing studies (Pace et al, 1989). Urea is another denaturing agent frequently used in protein unfolding studies. Denaturing agents unfold proteins due to disruption of noncovalent bonds and thereby inactivate the protein (Stryer, 1988). A polypeptide chain, without intramolecular crosslinks, will assume a random coil configuration in high concentrations of a denaturing agent. A crosslinked enzyme will be better able to withstand the action of the denaturing agent. Urea and guanidine hydrochloride unfolding are more likely than thermal unfolding to be completely reversible. The reconstitution of denatured proteins is often studied in conjunction with unfolding. Treatment of native and NHS-modified HRP with 3M guanidine chloride did not have any adverse effects on activity or thermal stability for any of the samples. The thermal profile seen in Fig. 4.27 has not been affected by the presence of 6M GnCl.

After overnight incubation of GnCl with HRP and NHS samples, 2-mercaptoethanol was added to samples as described in Section 2.15.7. An activity check on these samples after overnight incubation revealed that samples that had been treated with both GnCl and 2-mercaptoethanol were totally inactivated. However, HRP that had been treated with 2-mercaptoethanol alone (i.e. no GnCl) was fully active. 2-Mercaptoethanol acts to reduce disulphide bonds. Disulphide bonds present in HRP were cleaved by reduction with mercaptoethanol, but this reduction alone has no adverse effect on enzyme. Reduction alone is not sufficient to cause inactivation. However, when disulphide bonds were reduced and when the protein was subjected to a denaturant, all enzyme activity was lost. Modified HRP is no more resistant to inactivation than is native HRP. The modified enzyme may have unfolded enough to lose activity but not as far as a random coil configuration. It is possible to have an inactive, partially unfolded modified form. The native enzyme may be totally unfolded. Elaborate techniques are needed to determine if the modified protein is partially or totally unfolded. Apparently, any NHS ester crosslinks present did not

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prevent unfolding.

4.4.3.   Summary:

Horseradish peroxidase was modified with two homobifunctional N-hydroxysuccinimide esters, suberic acid ester and ethylene glycol ester. Successful crosslinking was seen using 1mg N-hydroxysuccinimide ester in 1.0 mg/ml HRP pH 8.0. This crosslinking enhanced the thermostability of HRP to a great extent. A large % of activity of modified samples remained after a 60 minute incubation at 72.5 °C. After 60 minutes incubation at 72.5 °C, 6.4% and 22.2% of activity that was present at t = 0 was lost for SA-NHS and EG-NHS, respectively. The modification effect persisted for up to 14 days. Characterization of modified enzyme samples was performed to determine if any properties, other than thermostability, had been altered. No oligomers were detected and hence there were no alterations in molecular weight of the modified enzymes. There were no discernible alterations in pH optimum, absorption spectra or resistance to denaturing agents. Slight differences observed in fluorescent emission spectra could not be related to modification. Overall, N-hydroxysuccinimide modification was very successful. The derivatives produced have potential use in industrial and clinical situations where elevated temperatures are used. Further investigations and optimization of procedures would determine if such derivatives could be used in organic solvents, at extreme pH values and in other novel situations.

4.5.   Heterobifunctional Modification of Horseradish Peroxidase:

4.5.1.   Introduction:

Heterobifunctional crosslinkers are bifunctional protein modification reagents having two different functional groups on the same molecule. Use of heterobifunctional crosslinkers allows two proteins to undergo crosslinking in a step-wise manner. Sequential crosslinking is useful because it is more specific than other methods and reduces the occurrence of unwanted side reactions. Reaction of horseradish

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peroxidase with heterobifunctional reagents was performed in order to form crosslinks that would increase the thermostability of the enzyme. The heterobifunctional reagents used were γ-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS) and 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (4NM). The structures for these are shown in Fig. 4.33 (a) and 4.33(b).
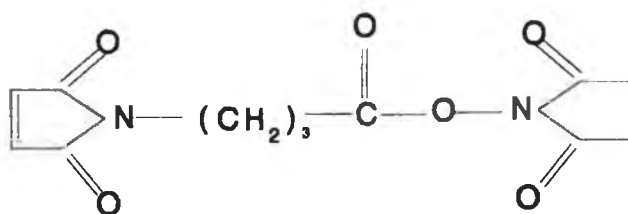
Fig. 4.33(a)   Structure of γ-maleimidobutyric acid N-hydroxysuccinimide ester.
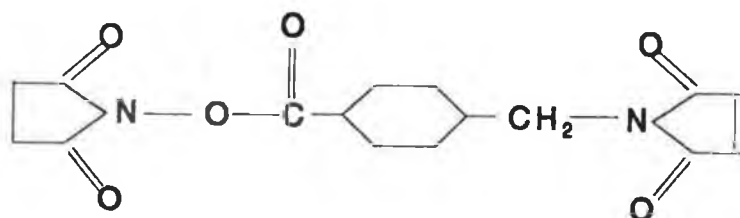
Fig. 4.33(b)   Structure of 4-(N-maleimidomethyl)-cyclohexane -1-carboxylic acid N-hyroxysuccinimide ester.

These two heterobifunctional reagents are specific for amino (succinimidyl portion) and thiol (maleimidyl portion) groups. Introduction of thiol groups to HRP was

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performed using 2-iminothiolane, which is specific for free amino groups. The reaction of 2-iminothiolane may be followed using the Ellman assay for thiol group determination. The presence of modified HRP forms following heterobifunctional treatment was assessed by studying the thermostability of HRP.

4.5.2. Results and Discussion:

4.5.2.1. Introduction of Thiol Groups into Horseradish Peroxidase:

An attempt to introduce sulphydryl (or thiol) groups into HRP using 2-iminothiolane was performed as described in Section 2.16. The structure of 2-iminothiolane is shown in Fig. 4.34. 2-Iminothiolane (2-IT) is also referred to as Traut's reagent and was formerly called methyl 4-mercaptobutyrimidate.

$$H_2C - CH_2$$
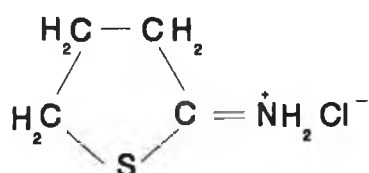$$H_2C \qquad\qquad C = \overset{+}{N}H_2 \; Cl^-$$
$$S$$

Fig. 4.34.   Structure of 2-Iminothiolane.

2-IT is a water soluble solid that reacts with primary amines on proteins at pH 7.0 - 10.0 without eliminating the charge on the amino groups (Means & Feeney, 1990). Lysine residues or N-terminal amines on proteins supply the primary amines. The lysine amino groups react with 2-IT to form amidine derivatives containing sulphydryl groups (Kenny et al, 1979). Crosslinking via disulphide bonds between adjacent sulphydryl groups can be promoted if the modified protein is subjected to oxidation with hydrogen peroxide (Traut et al, 1989). Oxidation of 2-IT modified HRP was not performed as any introduced free sulphydryl groups are needed for reaction with the heterobifunctional crosslinkers. Disulphide bond formation can be avoided if 0.1M EDTA is used in reaction solutions. This will chelate any oxidizing metals present in any of the solutions. The reaction of 2-IT with primary amines to introduce sulfhydryl groups is shown in Fig. 4.35.
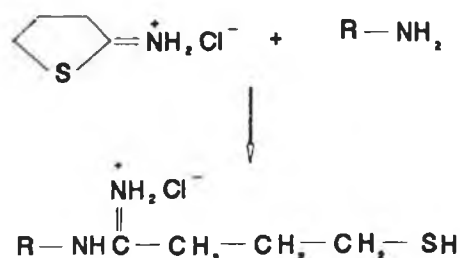
$$R-NHC-CH_2-CH_2-CH_2-SH$$

Fig. 4.35.   Reaction of 2-IT with primary amines.

After 2-IT reaction HRP should contain free amino groups and free sulphydryl groups. Free sulphydryl groups should be present and bound via lysines on HRP. The number of free sulphydryl/thiol groups on 2-IT modified HRP can be assessed by the Ellman assay as described in Section 2.18. 2-Iminothiolane treated samples were centrifuged to remove unbound 2-IT as described in Section 2.18 and assayed for thiol group number. Absorbance values for all samples were similar. It is not clear from Ellman assay results if any 2-IT has bound. Perhaps the Ellman assay is not sensitive enough to pick up extra thiol groups present. 2-IT treated HRP was subsequently modified with heterobifunctional crosslinking reagents.

4.5.2.2.   Heterobifunctional Modification of Horseradish Peroxidase:

The heterobifunctional reagents GMBS and 4NM were used to introduce crosslinks in 2-iminothiolane treated HRP, as described in Section 2.17. Heterobifunctionals bring about acylation of amino groups on proteins. This occurs via the NHS ester portion of the molecule. The rest of the crosslinking reaction involves the formation of a thioester by addition of a thiol group from the protein in question to the double bond of the maleimide group of the heterobifunctional (Han et al, 1984). Reaction of the thiol-specific end of both the heterobifunctionals was performed initially. Ellman's assay for thiol group determination (as described in Section 2.18) was used to assess if any heterobifunctional molecules had bound. A decrease in thiol group number would indicate that heterobifunctional molecules had reacted. No difference

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in thiol group numbers was discernible for any of the samples. Next, reaction of the amino specific end was performed. It is hoped at this stage that the thiol-specific end of the heterobifunctionals has attached to the introduced sulfhydryl groups on HRP. The amino specific end will be free to react with available lysines on the same or other enzyme molecules. After the reaction, crosslinks should be present between sulfhydryl and amino groups in the same or adjacent proteins. A check on initial recoveries after all above reactions were completed indicates that samples have lost 50% activity. This is a large loss in activity. The decrease may be due to the centrifugation procedures that led to a dilution of samples and hence to a loss of activity. The heterobifunctional reagents have an adverse effect on HRP activity. Thermal denaturation of native and heterobifunctional modified HRP was studied at 72.5°C. No enhanced thermostability was seen. Hence, it is evident that no beneficial crosslinking has occurred.

Heterobifunctional crosslinking reagents are frequently used in the preparation of immunoconjugates. They are often used in conjunction with 2-iminothiolane (Means & Feeney, 1990). The use of a protein modified with 2-iminothiolane in reaction with a heterobifunctional crosslinker allows two proteins, neither of which may contain free sulfhydryls, to undergo crosslinking in a stepwise manner. Yoshitake et al (1982) prepared an antibody - enzyme conjugate using a heterobifunctional to link the two molecules. The conjugation procedure was mild and efficient and mainly monomeric conjugates were formed. Fujiwara et al (1981) used heterobifunctional crosslinking reagents to prepare a homogeneous, hydrophobic hapten for use in the raising of specific antibodies. Recently, the heterobifunctional crosslinker GMBS and other heterobifunctionals have been used in immobilization of enzymes onto silicon supports, specifically for use in bioassays and biosensors (Rusin et al 1992, Eigler et al 1991).

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4.5.3.   Summary:

Horseradish peroxidase was modified with GMBS and 4NM, two heterobifunctional protein modification reagents. 2-Iminothiolane was used to introduce sulfhydryl groups into the HRP molecule. Ellman's assay for thiol group determination could not detect any increased levels of thiol groups in HRP after the 2-iminothiolane reaction. Reaction of the heterobifunctional reagents with the HRP (thiol and amino groups) resulted in a decrease in enzyme activity and no enhanced thermostability.

4.6.   Conclusion:

The objective of chemical modification studies involving horseradish peroxidase was to produce a thermostable derivative of the enzyme. This was successfully achieved for some of the chemical crosslinkers used, namely the bis-imidates and the NHS esters. A very dramatic increase in thermostability was seen in the case of the NHS esters. The chemical modification procedures that produced stabilized derivatives affected the protein part of the enzyme only. This leaves the carbohydrate portion unmodified. The carbohydrate portion is therefore free and can be used as a target for immobilization procedures and in biosensor construction. The fact that the carbohydrate is not modified in any of the methods used here is an important advantage over other procedures. The work presented here has future potential in many areas. Enzymes with enhanced stability can be used in industrial applications, in biosensors and in clinical assays and reagents. Stabilized enzymes have the advantages of longer life, wider applications and less limitations than other, less stable enzyme forms. Thus, stabilized horseradish peroxidase can be considered for a wide number of future developments. The work presented here is a step towards limitless use and application of enzymes in existing and novel situations.

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