A BIOCHEMICAL AND IMMUNOLOGICAL STUDY OF HORSERADISH PEROXIDASE

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DECLARATION

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ABSTRACT

This study describes:

a) the isolation and purification of horseradish peroxidase isoenzymes from horseradish roots,

b) the characterization of various forms and components of the enzyme by cation-exchange and reversed-phase high performance liquid chromatography,

c) the preparation of antibodies against horseradish peroxidase isoenzymes,

d) immunological studies for the development of an isoenzyme quantification method and

e) the formation of an enzyme-melamine conjugate for use in a melamine quantification immunoassay.

OPSOMMING

Hierdie studie beskryf:

a) die isolering en suiwering van peperwortel-peroksidase-isoënsieme vanuit die peperwortel,

a) die karakterisering van verskillende vorme en komponente van dié ensiem deur katioon-uitruilings en omgekeerde-fase *HPLC*

b) die voorbereiding van teenliggaampies vir peperwortel-peroksidase-isoënsieme,

c) immunologiese studies vir die ontwikkeling van ’n isoënsiem-kwantifiseringsmetode; en

d) die vorming van ’n ensiem-melamien-konjugaat vir gebruik in ’n melamien-kwantifiseringsmetode.

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God for the most amazing journey.

Vir my ouers

TABLE OF CONTENTS

ABBREVIATIONS

ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AtP-Z	A specific isoenzyme of *Arabidopsis thaliana*
CM	Carboxymethyl
Con-A	Concanavalin A
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
endo H	Endo-*ß-N*-acetylglucosaminidase H
FDNB	Fluorodinitrobenzene
Fuc	Fucose
GlcNAc	N-acetylglucosamine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HRP-C*	Recombinant horseradish peroxidase isoenzyme C
HRP-Z	A specific isoenzyme of horseradish peroxidase (where Z = A1-A3, B1-B3, C1, C2, D, E1-E6, N)
IAA	Indole-3-acetic acid
IEF	Isoelectric focusing
IgG	Immunoglobin G
LC/MS	Liquid chromatograph/mass spectrometry
Man	Mannose
Mr	Molecular mass
NB	Naked bacteria
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
pI	Isoelectric point
RZ	*Reinheitszahl*
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
TEMED	N,N,N,N-tetramethylethylenediamine
TFA	Trifluoric acid
Xyl	Xylose

1. INTRODUCTION

Horseradish peroxidase (HRP), a heme-containing glycoprotein, is one of the most studied peroxidases due to its widespread commercial applications. The primary source of HRP is the root of the horseradish, *Amoracia rusticana.* The horseradish is a member of the *Cruciferae*, a plant family of considerable economic importance. Horseradish roots are cultivated mainly for their culinary value and high peroxidase content.

HRP, a single polypeptide, contains iron(III)protoporphyrin IX and is classified as a class III plant peroxidase. The cyclic reduction and oxidation of the heme group in the presence of hydrogen peroxide gives rise to the enzyme's ability to oxidize a wide variety of organic and inorganic compounds. In addition to the heme group, HRP also contains calcium binding sites located at positions distal and proximal to the heme plane. Branched heptasaccharides on the surface of the enzyme account for approximately 22% of its molecular weight.

HRP exists as group of distinctive peroxidase isoenzymes which are classified into three major groups based on their respective isoelectric points. The most abundant isoenzyme, HRP-C, accounts for more than half of peroxidase activity in horseradish roots and is used in most clinical and analytical applications. The HRP-C content of raw material therefore determines the commercial value of a batch of roots. At present, there is no cost-effective method to determine HRP-C content of raw material or monitor HRP-C levels during initial purification steps. Production of HRP occurs on a relatively large scale and the inability to determine the commercial value of roots prior to processing leads to substantial financial losses for peroxidase manufacturers.

This study aimed to isolate and purify HRP-isoenzymes, specifically HRP-C, in order to investigate possible methods for the quantification of isoenzymes in raw material. For this purpose, different forms and components of HRP were characterized using cation-exchange and reversed-phase high performance liquid chromatography (HPLC) methods. Purified isoenzymes were also used in immunological investigations of the enzyme. In addition to characterization of HRP, the use of HRP in a commercial immunoassay for the detection of melamine was also investigated.

An overview of the general features of the enzyme is given in chapter two. The isoenzyme groups of HRP are also discussed in more detail in this chapter. Chapter three focuses on the catalytic mechanism of HRP as well as the physiological functions and commercial applications of the enzyme. In chapter four the developments in recombinant studies of the enzyme in comparison with other members of the plant peroxidase family are briefly summarised.

Current industrial methods were adapted for isolation and purification of HRP and these methods are explained in chapter five. Various forms and components of HRP are characterized by HPLC in chapter six. In this chapter the use of HPLC as an isoenzyme-quantification method was also investigated. The immunological studies of HRP-isoenzymes, more specifically, the preparation of antibodies against purified HRP-isoenzymes and the use of these antibodies in the detection of isoenzymes in primary root extracts, are described in chapter seven.

The commercial application of HRP in a melamine detection kit is examined in chapter eight. The aim of these experiments was to couple melamine to HRP for use in an immunoassay. The results obtained in this study are discussed in chapter nine and the experimental methods are described in detail in chapter ten.

2. MOLECULAR PROPERTIES OF HRP

2.1 Introduction

HRP [EC 1.11.1.7] is a glycosylated enzyme that contains a prosthetic heme group and binds two calcium ions. Both heme and calcium centres are essential for the structural and functional integrity of the enzyme [1]. The amino acid sequence, essential amino acids and three-dimensional structure of HRP will be reviewed in this chapter. The carbohydrate content, calcium binding centres, heme group as well as the contribution of these components to the structure:function of HRP will also be discussed.

HRP exists as a group of enzymes or isoenzymes as they are commonly referred to. These isoenzymes are classified into three major groups based on their respective isoelectric points (pI): acidic (A), neutral (B and C) and basic (D and E) [2,3]. The most abundant isoenzyme, neutral isoenzyme C, accounts for 50% of peroxidase activity in horseradish roots and is used in most commercial applications [4]. In this review HRP-C will serve as an example to illustrate the molecular properties of horseradish peroxidase. Comparisons between the three isoenzyme groups will conclude this chapter.

2.2 Primary structure

The complete amino acid sequence of HRP-C was determined in 1976 using classical methods (figure 2.1) [5]. HRP-C is a single polypeptide consisting of 308 amino acid residues with a molecular mass of 33 890 in its deglycosylated form. A pyroglutamate residue blocks the N-terminal. The C-terminal residue is more diverse, where some enzyme molecules lack the terminal-C residue, Ser308 [1]. The heterogeneity of the C-terminal is presumably determined chemically rather than genetically. A buried salt bridge is formed between Asp99 and Arg123. Four disulphide bridges occur between cysteine residues 11-91, 44-49, 97-301 and 177-209 [5].

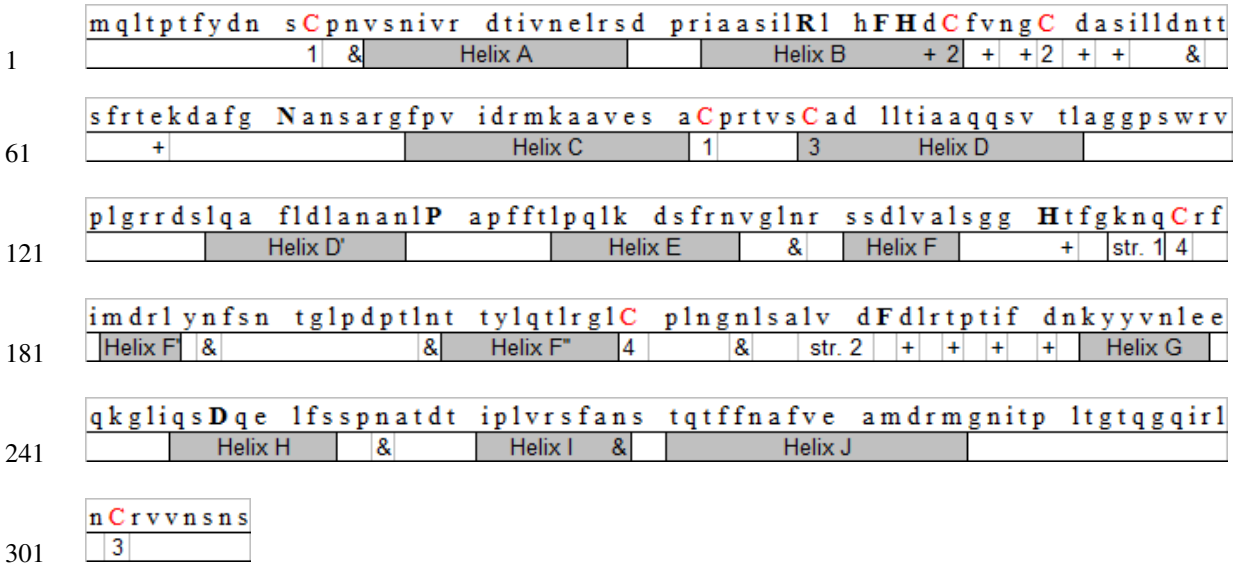

Figure 2.1. Amino acid sequence and secondary structure for HRP-C. *Uppercase bold residues*; residues important in either the distal or proximal heme pocket. *Residues indicated in red*; cysteine residues. *Line below amino acid sequence*; secondary structure. "&"; *N*-linked glycan attachment site. *"+"*; calcium ligand. *Numbers in second line*; disulphide pairings. Note that the amino acid sequence numbering should be read from the left. Adapted from [1].

2.2.1 Essential amino acids

Several amino acid residues that are essential to the functioning of HRP have been identified. Essential residues in the distal region of HRP-C, i.e. the region of the enzyme lying above the plane of the heme when viewed in the usual orientation, include His42, Arg38, Asp70 and Phe41 (figure 2.2). The distal histidine and arginine residues are conserved in all members of the plant peroxidase superfamily [1]. Both these residues are essential to the binding and stabilization of aromatic substrates and ligands. Furthermore, both His42 and Arg38 play a role in the formation and stabilization of compound I, a high oxidation state of the enzyme formed during the catalytic cycle. His42 accepts a proton from hydrogen peroxide and is therefore essential to compound I formation. Arg38, in turn, helps to stabilize a charged intermediate during compound I formation [6]. The catalytic cycle of HRP-C is discussed in more detail in chapter 4.2.

Site-directed mutagenesis studies have assessed the relative contributions of heme pocket residues to the rate of compound I formation (table 2.1). The rate of compound I formation is decreased by five to six orders of magnitude when His42 is mutated to Leu or Ala, respectively [7,8]. Catalytic activity of His42-mutants can be recovered in part by provision of a "surrogate" histidine residue or

by addition of 2-substituted imidazoles [9]. A precise structural location for this site relative to the heme iron is however not essential to achieve an intermediate rate of compound I formation. His42Glu and His42Asp mutants show intermediate rates of compound I formation, therefore suggesting that these residues act as alternative proton acceptors. His42Glu mutants show improved peroxygenase activity compared to wild-type HRP-C. This can be attributed to the increased accessibility of the distal cavity of the mutant form [10].

All class III peroxidase structures solved to date, except that of barley-derived peroxidase, have a conserved hydrogen bond network extending from His42 to the distal calcium binding site through Asn70 and Glu64 (see chapter 2.5) [11,12,13]. Asn70 and Glu64 facilitate the hydrogen bond network and are highly conserved among plant peroxidases. Asn70 maintains the basicity of the His42 side-chain through a hydrogen bond between the amide oxygen from Asn70 and the imidazole NH from His42 (figure 2.2) [4]. The basicity of His42 is decreased in Asn70Asp and Asn70Val mutants and affects not only the rate of compound I formation but also the rates of reduction of compounds I and II (table 2.1) [14].

Arg38 is essential in His42Glu mutants to retain an intermediate rate of compound I formation. This can be seen from the double mutant His42Glu:Arg38Ser that shows a decrease two orders of magnitude in the rate of compound I formation. Arg38 is not, however, absolutely essential for compound I formation but does promote proton transfer to the side chain of His42. This stabilizes the transition state for the heterolytic cleavage of the peroxide O-O bond [7]. In the absence of Arg38, the rate of O-O bond cleavage slows sufficiently for an intermediate, compound 0, to accumulate [15]. Theoretical calculations of electronic structure and spectra predict that compound 0 is an Fe(III)-OOH complex [16]. His42 and Arg38 are also important in the reduction of compounds I and II, although their precise roles are still under investigation.

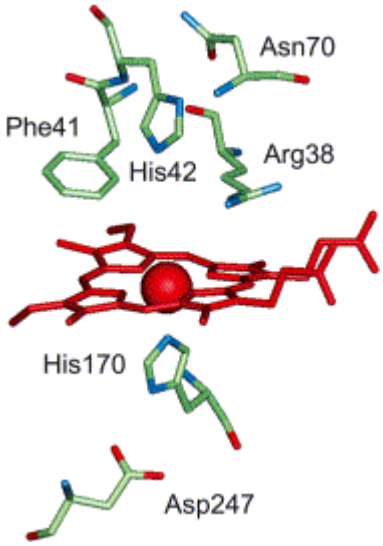

Figure 2.2. Key amino acid residues in the heme-binding region of HRP-C. *Red*; heme group and heme iron atom. *Atom colours*; amino acid residues [4].

Table 2.1. Summary of rate constants compound I formation in wild type (WT) HRP-C and selected site-directed mutants (one-letter abbreviations of amino acid residues), according to the following scheme [1]:

$$E + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} [E\text{-}(H)OOH] \xrightarrow{k_c} \text{compound I} + H_2O$$

HRP-C	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	k_c (s^{-1})
WT	1.7×10^7	$\approx 3.0 \times 10^{-1}$	$\approx 1.0 \times 10^3$
R38A	8.0×10^4		
R38H	1.3×10^6		
R38K	4.0×10^4	$\approx 2.0 \times 10^{-1}$	$>5.0 \times 10^2$
R38L	1.1×10^4		$\approx 1.4 \times 10^2$
F41V	2.1×10^6		
H42A	1.9×10^1		
H42A:F41H	3.0×10^4		
H42A:N70D	3.7×10^1		
H42D	1.3×10^3		
H42E	4.9×10^3		
H42E:R38S	3.2×10^1		
H42L	1.4×10^2	$\approx 8.0 \times 10^{-2}$	$\approx 7.0 \times 10^{-2}$
H42Q	9.6×10^1		
H42V:R38H	1.6×10^3		
E64G	4.3×10^5		
E64P	4.4×10^5		
E64S	4.3×10^5		
N70D	1.5×10^6		
N70V	6.0×10^5		
H170A	1.6×10^1		

The most important residue in the proximal region, i.e. the region of the enzyme below the heme-plane when viewed in the usual orientation, is His170. This proximal histidine residue binds covalently to the heme iron atom. Other residues of interest in the proximal region include a conserved aspartic acid residue, Asp247. The carboxylate side chain of Asp247 is hydrogen bonded to His170 Nδ1H and the strength of this interaction controls the imidazolate character of the His170-ring (see chapter 2.6) [17].

An interesting feature in the reduction of compounds I and II is the participation of Pro139. Pro139 is part of a structural motif which is conserved in plant peroxidases and crystallographic evidence indicates that Pro139 has a role in substrate oxidation and binding. For HRP-C this structural motif is Pro139-Ala140-Pro141. The carbonyl group of Pro139 acts as a hydrogen bond acceptor for a conserved water molecule, which also acts as a channel for proton transfer. The process of proton transfer is coupled to electron transfer from the substrate to the exposed heme edge [1].

Other essential amino acids include Phe41, Phe142 and Phe143. Phe41 prevents substrate access to the ferryl oxygen of compound I. Phe142 and Phe143 are part of the ferulate binding site of HRP. Additionally Phe142, together with Arg38, Phe68 and Phe179, determines the general electrostatic character of the substrate access channel [1]. Analysis of Phe179 mutants has confirmed it as the critical residue in the substrate access channel [13]. The substrate access channel is described in more detail in the next section.

2.3 Secondary and tertiary structure

The secondary structure of HRP-C is mainly α-helical with a small ß-sheet region (figure 2.3). HRP-C contains 10 prominent α-helices, termed helices A-J. These helices occupy similar positions in all the peroxidase structures that have been determined for the plant peroxidase superfamily (see chapter 4.4). Thirteen structurally conserved regions, with root mean square deviation of 2.5 Å with respect to the Cα backbone atom positions of the other known peroxidase structures, have also been identified for HRP [13]. In addition to the core peroxidase fold, HRP-C contains three helices denoted D´, F´ and F″ that are not found in class I or II peroxidases. Helices F′ and F″ are situated in a long insertion between helices F and G. This insertion displays considerable variation among class III peroxidases in both amino acid sequence and number of residues [18]. A disulphide bridge between Cys177 and Cys209 maintains the overall structural integrity in this region [4]. Helix F′ provides additional contacts to the substrate access channel [19]. Some of these contacts modulate

the aromatic donor molecule binding. It is speculated that this region of the enzyme structure is involved in the retention or stabilization of radicals formed by the enzyme [13].

2.3.1 Substrate access channel

Nuclear magnetic resonance (NMR) spectra have long suggested two Phe residues situated towards the entrance of the aromatic donor-interaction site [20]. Studies have confirmed the inner channel and lining of the heme cavity has an overall positively charged character primarily due to Arg38. It has also been shown that the peripheral layer of the channel has a hydrophobic character due to the contribution of Phe68, Phe142, Phe179 and the heme C18 methyl. These Phe residues form a distinctive hydrophobic patch near the exposed heme edge. Phe179 is positioned close to the heme edge and sterically impedes channel access. This region is responsible for the superior ability of HRP-C to form stable 1:1 complexes with reducing substrates [13].

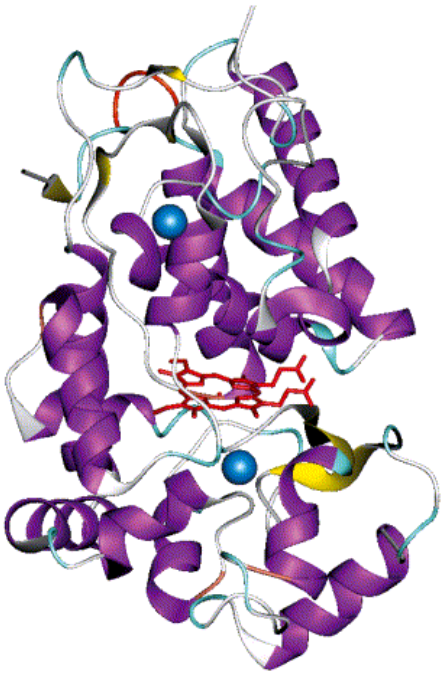

Figure 2.3. Three-dimensional representation of the X-ray crystal structure of HRP-C. F' and F'' α-helices appear in the bottom right-hand quadrant of the enzyme molecule. *Purple*; α-Helical regions. *Yellow*; β-sheet regions. *Red*; the heme prostetic group located between the proximal and distal domains. *Blue spheres*; calcium atom [4].

2.4 Carbohydrate component

Carbohydrate residues form an integral part of HRP. The molecular mass for glycosylated HRP-C determined by mass spectrometry ranges from 42 200 – 44 000 Da [21]. The total carbohydrate content is dependent on the source of the enzyme and carbohydrate residues account for 22-27% of the molecular mass of HRP-isoenzymes. The carbohydrate content for HRP-C typically ranges between 18 and 22% of the molecular mass [22].

Nine potential glycosylation sites have been identified for HRP-C and are recognised in the amino acid sequence from the motif Asn-X-Ser/Thr where 'X' represents any amino acid residue except Pro (figure 2.1). Eight of the nine potential glycosylation sites are exposed on the surface of the enzyme and are occupied by carbohydrate chains [5]. The glycosylation sites are in loop regions at Asn13, Asn57, Asn158, Asn186, Asn198, Asn214, Asn255 and Asn268. The non-glycosylated site is found at residues 286-288 and appears to be inaccessible to the glycosyl transferase which initiates carbohydrate synthesis [23]. Trypsin digests of HRP-C give rise to 28 tryptic peptides of which seven are glycopeptides. Two of the tryptic glycopeptides form inter-residue disulphide bonds with other tryptic peptides while one of the other tryptic glycopeptide forms an intra-residue disulphide bond. Asn186 and Asn198 are part of a doubly glycosylated peptide. Asn57 and Asn267 were found to be more heterogeneously glycosylated than the other sites [24].

The type of carbohydrate structure at any of the eight glycosylation sites can vary and creates a heterogeneous carbohydrate profile for HRP-C. This heterogeneity can be explained in terms of combinations of the four basic carbohydrates detected at all the asparagines-glycosylation sites. The asparagine-linked carbohydrates are composed of monosaccharides mannose (Man), N-acetylglucosamine (GlcNAc), xylose (Xyl) and fucose (Fuc) [21,24]. Purified HRP-C contains 2.6 mol Man, 2 mol GlcNAc and 0.8 mol each of Xyl and Fuc, therefore an approximate molar ratio of 3:2:1:1. Deviations from this ratio indicate the degree of heterogeneity of the carbohydrates attached to the each of the glycosylation sites [22].

The major carbohydrate, branched heptasaccaride Manα3(Manα6)(Xylß2)Manß4-GlcNAcß4(Fucα3)GlcNAc, is characteristic of glycoproteins and accounts for 75-80% of the carbohydrate content for HRP-C (figure 2.4) [25,26]. Many other minor oligosaccharides have also been characterized but little is known about the distribution of these minor carbohydrates along the peptide chain. The minor carbohydrate residues of HRP generally contain several Man residues and

two terminal GlcNAc. These minor carbohydrate residues belong to the $Man_mGlcNAc_2$ (m = 4 to 7) and $(Xyl)_xMan_m(Fuc)_fGlcNAc_2$ (x = 0 or 1; m = 2, 4, 5, 6; f = 0 or 1) families [22].

The heterogeneity of the carbohydrate profile for HRP-C as well as the high carbohydrate content of the enzyme prevents suitable crystal formation for X-ray crystallography. Deglycosylated HRP has a decreased solubility in salt solutions and might therefore be more suitable for the growth of crystals. Removal of carbohydrates by enzymatic hydrolysis has proven unsuccessful. Mild chemical deglycosylation can be used to produce a homogeneous and fully active enzyme, although this enzyme still contains traces of carbohydrates. For example, mild chemical deglycosylation using anhydrous trifluoromethanesulfonic acid removes all carbohydrates except for two GlcNAc residues at each site [21]. This method yields a fully active, homogeneous deglycosylated enzyme that can be recovered after ion-exchange chromatography. This deglycosylated enzyme shows a decreased solubility when compared to native HRP-C.

The role of carbohydrates in determining the physicochemical properties of HRP is still unknown. It seems unlikely that the carbohydrate moieties participate directly in catalytic activity since many other hemoproteins possess peroxidase activity in the absence of carbohydrates [2]. Some controversy exists in the literature regarding the activity of deglycosylated HRP. In 1990, Silva *et al*. reported that deglycosylated HRP showed two orders of magnitude lower specific activity than the glycosylated form of the enzyme [27]. Two other studies, one with chemically deglycosylated HRP and the other with non-glycosylated recombinant HRP, showed, however, that more than 50% of the activity of glycosylated HRP is maintained [28]. It therefore appears that carbohydrates do not interfere with substrate access or substrate binding in HRP. Furthermore, HRP and deglycosylated HRP have identical absorption spectra in the range of 250-700 nm. This indicates that the heme environment remains unchanged by deglycosylation through chemical treatment. The specific peroxidase activity as well as reaction kinetics are also identical for both glycosylated and deglycosylated forms of HRP [21].

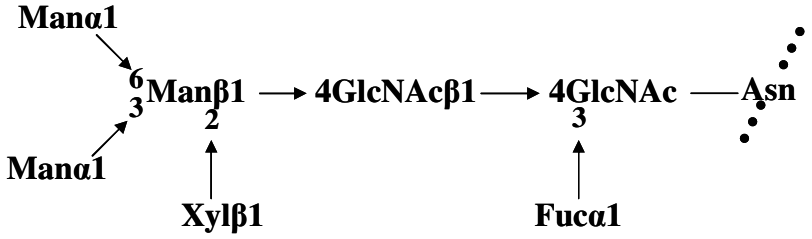

Figure 2.4. Major carbohydrate for HRP-C: Manα3(Manα6)(Xylß2)Manß4-GlcNAcß4(Fucα3)GlcNAc [4].

All glycosylation sites point away from the enzyme and are evenly distributed over the entire surface. This has lead researchers to believe that the main role of carbohydrates is to increase the solubility of HRP in water. Non-glycoslylated recombinant HRP shows enhanced sensitivity to high H_2O_2 concentrations, suggesting that carbohydrates may also increase the enzyme's resistance to free radical-induced protein cross-linking [13]. It appears that the eight neutral carbohydrate chains of HRP-C also stabilize the enzyme towards the action of proteolytic enzymes [29]. The carbohydrates of HRP are resistant to endoglycosidases under certain conditions. For example, the resistance of HRP to enzymatic hydrolysis of carbohydrates by endo-*β*-*N*-acetylglucosaminidase H (endo H) is consistent with the specificity of endo H, which reportedly only hydrolyzes high mannose N-glycans. The resistance of HRP is most likely due to the presence of Fucα1-3 at the innermost GlcNAc, a feature that is unique to plant carbohydrates [21].

2.5 Calcium binding centres

The proximal and distal regions of HRP each contain a calcium binding site (figure 2.3). The existence of two calcium binding sites may be the result of an early gene duplication event during the evolution of plant peroxidases [30,31]. The presence of calcium in HRP has been demonstrated by atomic absorption spectroscopy. Neutral HRP-isoenzymes reportedly contain one calcium atom at each site [32] and early studies revealed that HRP-C contains 2.0 ± 0.13 calcium per mole enzyme [33].

As discussed in chapter 2.2.1, a network of hydrogen bonds links the calcium binding sites to the heme-binding region. Both the distal and proximal calcium sites are seven-coordinate and the ligands are carbonyl and side chain oxygens. Distal *O*-donors are the side-chain carboxylates of Asp43 and Asp50, the hydroxyl group of Ser52, the carbonyl backbone of Asp43, Val46 and Gly48 and one structural water molecule, Wat15. Some of these calcium ligands stem from the loop region delineated by the Cys44-Cys49 disulphide bridge. The distal Ca^{2+} is structurally coupled to the active site through Asp43 which provides two ligands to Ca^{2+} [13]. Wat15, a ligand of the distal calcium site, is hydrogen bonded to Glu64 Oϵ1, which in turn shares an additional hydrogen bond with another structural water molecule, Wat14. Wat14 and the backbone carbonyl of Glu64 are hydrogen bonded to Nδ2H_2 Asn70, whereas Asn70 Oδ1 is bonded to His42 Nδ1H. Glu64 and Asn70, the residues that facilitate the connecting networks of hydrogen bonds, are highly conserved among plant peroxidases [18].

Site-directed mutagenesis has revealed that three HRP-C mutants, Glu64Gly, Glu64Pro and Glu64Ser, contain only one mol calcium per enzyme molecule [34]. The kinetic properties of these mutants are significantly different to those of wild-type HRP-C (table 2.1). A large decrease in k_1 is seen in these mutants, possibly due to the reorientation and decrease in basicity of His42 as well as the increase in the redox potential (Fe^{2+}/Fe^{3+}) of the mutants. NMR spectra for the mutants are remarkably similar, suggesting that replacement of Glu64 causes the electronic structure of the heme group to change. These changes are, however, independent of the nature of Glu64 substitution.

The proximal *O*-donor ligand set is provided by the backbone carbonyls of Thr171, Thr225 and Ile228, the side-chain carboxylates of Asp222 and Asp30 and the side-chain hydroxyls of Thr171 and Thr225 [4]. Thr171 is a Ca^{2+} ligand and indirectly couples the proximal calcium site to the heme group through side-chain linking with His170. With the exception of barley peroxidase, the spatial relationship between the calcium binding sites and the heme group appears to be conserved among class II and class III peroxidases [6].

The importance of both calcium binding sites to the structure:function of HRP-C is illustrated by the need to incorporate calcium in *in vitro* folding mixtures to obtain active recombinant HRP [28]. Calcium can be removed from native HRP by incubating the enzyme with guanidinum hydrochloride and ethylenediaminetetraacetic acid (EDTA) at neutral pH. Studies have shown differences between optical, NMR and electron paramagnetic resonance spectra of calcium-depleted HRP and the native enzyme [35]. The influence of calcium-depletion on the environment of the heme group has also been confirmed by crystallographic studies [13]. Calcium-depleted HRP-C shows reduced thermal stability and approximately 40% decrease in specific activity compared to native HRP. Calcium-depleted HRP-C can be successfully reconstituted on calcium addition [33]. Alternatively, the calcium ions of HRP-C can be substituted by either divalent metal cations (Ba^{2+}, Cd^{2+} and Sr^{2+}) or lanthanides (Ln^{3+}) without compromising the structural and functional integrity of the enzyme [36].

2.6 Heme prosthetic group

All HRP-isoenzymes contain a non-covalently bound ferriprotoporphyrin IX, or heme group, which forms the active centre of the enzyme (figure 2.5) [2,29]. Traditionally it was assumed that the iron atom in HRP-C was six-coordinate, with a water molecule occupying the sixth coordination position [37]. NMR and Raman spectroscopy have, however, shown that the Fe(III) of HRP-C in the resting state of the enzyme is a five-coordinate, high spin species [38]. Only small amounts of six-coordinate high-spin species can be detected in solutions and solid states of HRP-C [1].

The heme group is situated between the proximal and distal regions of the enzyme and the heme periphery is well exposed to solvents. Direct hydrogen bonds exist between the heme propionates and the enzyme through Arg38, Ser35, Ser73 and Gln176. As mentioned previously, Arg38 is a key catalytic residue and is conserved in the distal heme pocket of class III peroxidase. A hydrogen-bond network involving Arg38 couples the distal heme pocket to the proximal side. Arg38 is hydrogen-bonded to a heme propionate through Wat41. Gln176 connects the two halves of this extended hydrogen-bonding network above and below the heme group [13].

Ligand binding can occur at the two axial coordination sites, namely the fifth and sixth position. At the fifth, or proximal, position the heme group is attached to the apo-enzyme by a coordinate bond. For HRP-C this coordinate bond occurs between the heme iron atom and His170 side-chain Nϵ2 atom (figure 2.5) [4]. The sixth, or distal, position is vacant in the resting state but is available to H_2O_2 during enzyme turnover.

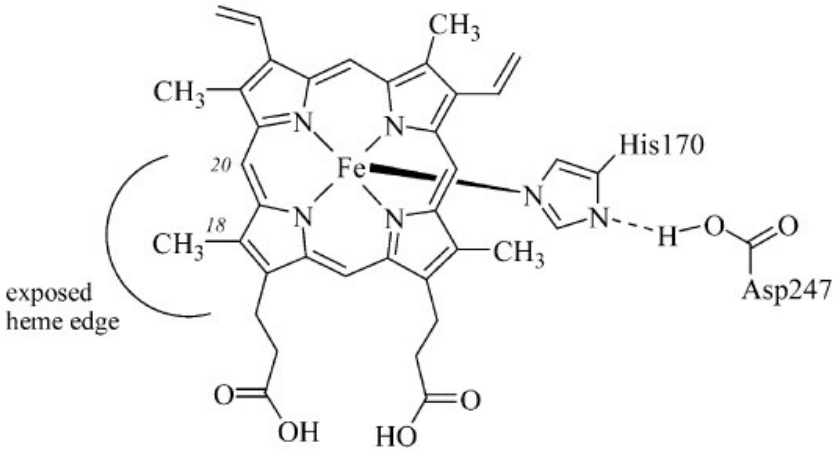

Figure 2.5. Heme group of HRP-C and coordinate bond between the heme iron atom and His170 [4].

As mentioned in chapter 2.2.1, the imidazolate character of the His170 ring is controlled by the carboxylate side-chain of Asp247. The presence of a strong hydrogen bond between Nδ1 His170 and Asp247 is believed to increase the basicity of the His170 proximal ligand. This helps to stabilize high oxidation state intermediates while maintaining the heme in its five-coordinate state. His170Ala mutants are still able to bind to the heme iron but the His170Ala-imidazole complex does not maintain the five-coordinate state and therefore full catalytic activity is not restored. Upon H_2O_2 addition His170Ala mutants undergo heme degradation and compounds I and II are not detected. This suggests that a major function of His170 is to "tether" the heme iron atom and prevent the formation of 6-coordinate states [8].

The coordination- and spin states of HRP are pH-dependent. Little change occurs in these states between pH 4 and 10. At pH 3.1 the resting state enzyme of HRP-C is a five-coordinate high-spin form in which His170 is replaced by a water molecule [39]. At high pH values the enzyme changes to a six-coordinate low-spin species known as the alkaline form [40,41]. This transition occurs due to the stabilization by the hydrogen bond between the hydroxide ligand and the Arg38 side chain. Some information is available for the resting states of HRP-A1 and HRP-A2; the resting states of the other isoenzymes have not yet been characterised [42].

2.6.1 Apo-peroxidase

Various heme dissociation methods resulting in the precipitation of apo-peroxidase have been reported. Precipitated apo-HRP can usually be separated from the heme-containing supernant by centrifugation. Apo-HRP is easily reconstituted into fully active HRP on addition of heme. When heme is added to any apo-HRP isoenzyme the resulting mixture of apo-enzyme and heme exhibits absorption properties characteristic of the specific isoenzyme from which the apo-isoenzyme was originally derived. Spectral studies have suggested a different mode of heme group attachment among the isoenzymes [2].

The renaturation of apo-HRP is dependent on the stability of two domains within the apo-enzyme. The two domains were identified by tryptic digests of apo-HRP and appear to be essentially conserved from the active enzyme. One domain is represented by tryptic peptide 160-224 (pT160-224) and contains the proximal iron-coordinate, His170. This peptide can bind heme specifically, although not as strongly as the entire apo-enzyme. The other domain consists of two peptides (pT1-

149 and pT265-308) that are intimately linked in space rather than being independent domains held together by a disulphide bridge [29].

The structures for holo- and apo-HRP have been studied by circular dichromism, tryptophan fluorescence and NMR [43-45]. These techniques have established certain conformational changes in the secondary structure of HRP after the removal of the heme group. Immunological studies have confirmed conformational changes in apo-HRP. These studies have shown apo-HRP to be more immunogenic than holo-HRP, indicating a partial unfolding of the apo-enzyme which makes immunogenic amino acids more accessible. Both N- and C-terminal regions for apo-HRP were also found to be more immunogenic and several epitopes that are specific only for apo-HRP have been identified. Some epitopes determined for holo-HRP were also detected for apo-HRP but with one or two amino acid shifts. The increased amount of antigenic epitopes for apo-HRP and partial shifts in the locations of these epitopes suggest a more flexible and less compact structure for apo-HRP. Therefore the role of the heme group seems to include keeping the two domains of the enzyme in close proximity and stabilizing of the enzyme [29].

2.7 Horseradish peroxidase isoenzymes

As mentioned in the introduction to this chapter, HRP exists as a group of isoenzymes. The existence of these isoenzymes was first noted in 1942 [46] and confirmed by later investigations [47,48]. Although controversy existed regarding the classification and properties of the isoenzymes during early investigations [49], at least fifteen HRP-isoenzymes have been isolated from horseradish roots to date [4]. Classification based on pI is now commonly accepted and is used to divide HRP-isoenzymes into three major groups, namely acidic (A), neutral (B and C) and basic (D and E) HRP [1]. To date three acidic (A1, A2, A3), five neutral (B1, B2, B3, C1, C2) and six basic (E1-E6) have been isolated and characterized (table 2.2) [2,3,32,50].

It is important to note that, although only fifteen HRP-isoenzymes have been isolated, up to 42 isoenzymes can be detected using isoelectric focusing (IEF) [46]. It is not known, however, whether these additional observed HRP-isoenzymes are in fact true allelic forms of HRP. Experimental conditions of the detection method could lead to deamidation of surface residues and cross-linking with phenols. These modifications could give rise to artefacts that appear as additional isoenzymes. Another possible explanation for the additional isoenzymes is posttranslational modification by C-terminal processing. For example, nonspecific proteolytic cleavage of an isoenzyme may produce a

variant lacking a C-terminal residue [1]. Heterogeneity within the isoenzyme groups may also be explained by functionally neutral, single-base mutations or carbohydrate heterogeneity originating from biosynthesis and degradation [5]. Although systematic surveys of *Arabidopsis thaliana* have revealed that it is possible for a single plant species to contain many true peroxidase isoenzymes [51-54], further investigation into the origin of HRP-isoenzymes is needed.

Table 2.2. Isoelectric points for horseradish peroxidase isoenzymes

Acidic[a]			Neutral[b]					Basic[c]						Apo[d]
A1	A2	A3	B1	B2	B3	C1	C2	E1	E2	E3	E4	E5	E6	
<5	3.50	<5	5.75	7.15	7.10	9.40	9.63	10.60	10.63	>12	>12	>12	>12	6.8

[a] Cited from Ref [55] [b] Cited from Ref [32] [c] Cited from Ref [50] [d] Cited from Ref [56]

2.7.1 Neutral isoenzymes

The five neutral isoenzymes are the most abundant form of peroxidase in horseradish. It is evident that the neutral isoenzymes occur naturally in horseradish roots although it is not yet known whether these isoenzymes are true allelic isoenzymes or products of post-translational modifications [32].

The physicochemical and chemical properties of the neutral isoenzymes are very similar. The molecular mass, rate constants of the catalytic reaction and molar absorption coefficient are essentially the same for the neutral isoenzymes. The neutral isoenzymes do differ markedly however in terms of pI. Isoelectric points for the group range from 5 to 10. The difference in pI can be attributed to small differences in the number of ionized groups on charged amino acids (i.e. aspartic acid, glutamic acid, lysine and arginine) [32].

The most abundant isoenzyme, HRP-C, accounts for 50% of the peroxidase activity in horseradish roots and holds the most commercial value. Before the amino acid sequence for HRP was determined many researchers had identified a form of HRP similar to what is now know as HRP-C. Shannon's isoenzyme C [2], Paul's isoperoxidase IIIb [49] and HRP-C1 isolated by Aibara *et al*. in 1982 [32] all match the isoenzyme sequenced by Welinder in 1979 [5]. This study will therefore refer to this isoenzyme simply as HRP-C. HRP-C was therefore the first HRP-isoenzyme to be sequenced and a complete tryptic map of HRP-C is also available [57]. The recombinant form of HRP-C has been studied extensively and site-specific mutagenesis has been used to investigate the

catalytic properties of this isoenzyme (see chapters 3 and 4). As mentioned earlier, HRP-C contains eight carbohydrate side chains, a prostetic heme group and two calcium ions. The molecular mass of the protein moiety is 33 890. The addition of the heme group and 2 Ca^{2+} ions increase the molecular mass to 34 590. In its glycosylated form the overall molecular mass of HRP-C is in the order of 44 000 [1].

Isoelectric points ranging from 8.8 to 9.6 have been reported for HRP-C [2,32,55,56]. The charged residues of HRP-C are equally distributed along the peptide chain. At neutral pH the net charge of the native enzyme is a calculated as +2. The 2 Ca^{2+} ions are essential to the resultant positive net charge. Apo-peroxidase has a lower pI-value than the native enzyme even though the heme group contributes a -1 charge to peroxidase (table 2.3). Although apo-HRP retains most of the native enzyme structure, its ability to bind calcium appears weaker and possibly permits the exposure of four more carboxyl groups in apo-HRP during pI determination [56].

2.7.2 Acidic isoenzymes

In 1966 Shannon *et al*. isolated and purified seven isoenzymes from a crude root extract by repeated ion-exchange chromatography [2]. These seven isoenzymes were categorized as three acidic (A1-A3), two neutral (B and C) and two basic isoenzymes (D and E). No inter-conversion among isoenzymes was observed during repeated purification steps and the authors therefore concluded that the observed isoenzymes were authentic isoenzymes rather that artefacts of purification procedures.

Following the initial identification of acidic isoenzymes, HRP-A2 has been the focus of most investigations. HRP-A2 is the most abundant acidic isoenzyme found in horseradish root and can also be purchased commercially. Although the amino acid sequence for HRP-A2 has been determined, crystallization has been unsuccessful to date [18,55].

The molecular properties of the acidic isoenzymes are notably different to that of the neutral isoenzymes. Investigations into the primary structure of the acidic isoenzymes have shown these isoenzymes contain significantly less arginine, methionine, tyrosine and phenylalanine residues than the neutral isoenzymes [2,50]. HRP-A2 consists of 305 amino acid residues and shows only 54% sequence identity to HRP-C [55]. HRP-A1 has been partially sequenced and is reportedly 80% identical to HRP-A2 [18].

Table 2.3. Chemical compositions of the neutral HRP isoenzymes. Amino acid, carbohydrate and calcium values presented as mol/mol of enzyme. Molecular mass presented as Da [32].

	B1	B2	B3	C1	C2	C*
Lysine	5.1	5.3	5.4	5.7	5.9	6
Histidine	3.0	3.2	3.0	3.1	3.2	3
Arginine	19.9	19.7	18.5	19.8	20.2	21
Aspartic acid	45.9	46.6	43.0	45.3	47.2	48
Glutamic acid	20.6	20.6	19.4	20.8	21.2	20
Glycine	17.9	17.0	15.9	16.9	17.1	17
Alanine	22.3	22.4	21.1	22.6	23.4	23
Valine	15.0	15.2	14.5	15.3	15.6	17
Leucine	33.0	33.4	32.1	32.1	33.3	35
Isoleucine	11.5	11.8	10.7	11.8	12.6	13
Proline	17.5	18.8	18.0	18.3	18.6	17
Serine	22.3	21.6	20.7	20.4	22.1	25
Threonine	22.9	23.1	21.8	23.4	23.9	25
Half-cystine	7.8	7.6	7.5	8.3	7.4	8
Methionine	3.8	3.7	3.6	3.9	3.6	4
Phenylalanine	19.0	19.2	18.2	19.2	19.7	20
Tyrosine	4.8	4.8	4.5	4.8	4.6	5
Tryptophan	1.1	1.3	1.1	1.3	1.1	1
Total	293.4	295.3	279.0	293.0	300.7	308
True sugars	31.9	34.9	31.3	35.8	33.0	
Glucosamine	13.3	12.6	12.4	13.6	12.1	
Calcium	2.1	2.1	2.0	2.0	2.3	2
Molecular weight	42 700	42 800	41 200	41 700	42 100	

*Determined by amino acid sequence

Differences in carbohydrate composition between the acidic and neutral isoenzymes can also be seen. The acidic isoenzymes have the highest carbohydrate content of all the isoenzyme groups. Galactose and arabinose are present in all three acidic isoenzymes but are absent in the neutral isoenzymes. Conversely, HRP-B and HRP-C contain xylose, fucose and galactosamine which are not detected for isoenzymes A1-A3. Both acidic and neutral groups contain mannose and monnosamine but test negative for sialic acid [2]. HRP-A2 has seven potential asparagine-glycosylation sites and a carbohydrate content of roughly 20%. The seven carbohydrates of HRP-A2 show the same structure and heterogeneity as the eight carbohydrates of HRP-C. The branched heptasaccaride, Manα3(Manα6)(Xylß2)Manß4-GlcNAcß4(Fucα3)GlcNAc is the major carbohydrate form for both HRP-A2 and HRP-C [50].

The acidic and neutral isoenzymes also differ in catalytic activity. The specific activity of HRP-A is generally lower than that of HRP-C. In spite of lower specific activity, HRP-A is less readily inactivated by peroxides. According to early studies, HRP-C and HRP-A contain 2.0 ± 0.13 and 1.4 ± 0.19 moles calcium per mole enzyme, respectively [33]. With the exception of an Ile/Ala substitution at position 228, the amino acid residues involved in calcium binding are identical for HRP-C and HRP-A2 [18]. When the calcium ions are removed, the specific activities of HRP-C and HRP-A are decreased by 40% and 15%, respectively. Only calcium-depleted HRP-C can be successfully reconstituted and HRP-C undergoes exchange with $^{45}Ca^{2+}$ while HRP-A does not. This supports the hypothesis that true structural differences exist between the calcium binding sites of the two isoenzymes [33].

2.7.3 Basic isoenzymes

Although the basic isoenzyme content in horseradish roots is relatively low, these isoenzymes can easily be purified from the extract by cation-exchange chromatography. Amino acid analysis indicates that the amino acid compositions of the basic isoenzymes differ, suggesting that the six isoenzymes may be allelic gene products and are not simply formed by posttranslational modifications. The basic isoenzymes can also be classified into three groups by amino acid and carbohydrate compositions. The first group is made up of E1, E2 and E5, the second group includes E3 and E4, and the third group E6. HRP-E5 shows the closest similarity to the amino acid composition of HRP-C. HRP-E5 consists of 306 amino acid residues and exhibits 70% sequence identity with HRP-C [58]. There is essentially no difference between the catalytic activity of the basic isoenzymes and that of HRP-C [50].

The basic HRP-isoenzymes contain significantly less carbohydrates that the neutral and acidic isoenzymes. Compared to HRP-C, the basic isoenzymes feature lower carbohydrate content and lower molecular mass. The considerable differences among the molecular mass of the six basic isoenzymes, can be attributed to the carbohydrate content since the molecular mass of the protein moieties are similar to one another (table 2.4). HRP-E1 and HRP-E2 contain relatively large amounts of carbohydrates, 12.8 and 14.1%, respectively, and are characterized by pI-values of approximately 10.6. HRP-E3 to HRP-E6 has extremely high pI-values of over 12 and considerably lower carbohydrate content ranging between 0.8 and 4.2%. It is thought that some of the basic isoenzymes exhibit only one site for carbohydrate attachment. Additionally, the carbohydrate chains that attach to these respective sites are shorter than the carbohydrates of HRP-C. HRP-E5 has only two glycosylation sites located at Asn188 and Asn214. These sites are analogous to the glycosylation sites of HRP-C found at Asn186 and Asn214, respectively [58,59]. Due to their relatively low carbohydrate content the basic isoenzymes are suitable for crystallization. Large single crystals of HRP-E4 are reportedly more suitable for further structural analysis than HRP-C crystals [50].

Considering the physicochemical differences of the isoenzyme groups as discussed in this chapter, it is not surprising that these groups also differ markedly in kinetic properties. While all HRP-isoenzymes appear to catalyze the same reaction, there is no doubt that the different isoenzyme groups serve specialized albeit unknown biological functions in the plant. The catalytic mechanism of HRP and its possible functions are reviewed in the following chapter.

Table 2.4. Chemical compositions of the basic HRP-isoenzymes. Amino acid residues and carbohydrates values presented as number of residues/mol. Molecular mass presented as Da [50].

	E1	E2	E3	E4	E5	E6
Lysine	6.7	7.3	5.6	5.2	7.3	7.2
Histidine	2.9	2.9	4.1	4.0	3.1	3.0
Arginine	21.3	21.2	24.0	24.5	25.7	22.7
Aspartic acid	44.7	43.8	38.0	37.5	44.4	41.5
Glutamic acid	21.6	21.7	20.9	20.9	16.2	16.9
Glycine	16.8	17.4	30.6	30.4	20.4	23.0
Alanine	23.8	23.4	23.0	22.7	25.6	32.1
Valine	19.1	19.1	28.1	28.2	20.0	19.0
Leucine	30.1	29.3	24.4	24.3	34.0	21.5
Isoleucine	13.1	12.6	15.4	16.0	14.2	15.7
Proline	17.5	17.5	14.8	14.4	19.2	11.4
Serine	23.2	20.2	22.8	22.8	25.4	29.2
Threonine	23.1	23.4	23.2	23.9	23.6	27.9
Half-cystine	8.4	8.4	8.1	7.9	7.8	8.1
Methionine	2.5	3.1	4.1	3.8	3.7	4.7
Phenylalanine	16.8	16.6	12.9	13.2	20.9	14.3
Tyrosine	5.4	5.9	2.1	2.1	5.3	4.1
Tryptophan	0.8	1.9	1.1	1.2	1.1	1.8
Total	297.8	295.7	303.2	303.0	317.9	304.1
Glucosamine	5.4	7.1	0.8	0.7	1.8	4.1
Hexose and pentose	25.7	26.9	0.8	0.8	7.9	1.8
Carbohydrate moiety	5 002	5 487	323.0	323.0	1 613	645.0
Protein moiety	32 843	32 630	32 314	32 402	34 804	32 701
Molecular mass	38 800	39 000	33 900	33 700	37 300	33 900

3. MECHANISM OF ACTION

3.1 Introduction

HRP is classified as an oxidoreductase due to the cyclic reduction and oxidation of the heme group which gives rise to its enzymatic activity. In brief, HRP reduces hydrogen peroxide to form a complex which can oxidize a variety of organic and inorganic substrates [21]. Substrates include amines, indoles, sulfonates, phenols and phenolic acids [4]. The majority of reactions catalyzed by HRP can be summarized by equation 1:

$$HRP + H_20_2 + substrate \leftrightarrow (HRP\text{-}H_20_2) + substrate \leftrightarrow product + HRP + 2H_20 \qquad (1)$$

This chapter will focus on the catalytic mechanism, functional roles and commercial applications of HRP. The specific contribution of structural components to the enzymatic activity of HRP is discussed in chapter 2.

3.2 Catalytic mechanism of HRP

The catalytic mechanism of HRP, specifically that of HRP-C, has been studied extensively [1,6] and can be seen as two one-electron reduction steps that generate radical species. The generation of radical species creates a complex profile of reaction products. Primary products (e.g. dimers) can also act as peroxidase substrates which in turn produce additional radical species that take part in further coupling reactions. As a result, oxidation of aromatic substrates by HRP often produces oligomers and polymers [1]. Although the two one-electron reduction steps describe most HRP reactions single two-electron reductions, including the oxidation of bisulfite ions [60] and iodide [61], have been reported.

Equations 2 – 5 represent the catalytic cycle of HRP and apply to most reactions the enzyme catalyzes, irrespective of the specific isoenzyme involved. The catalytic cycle of HRP is illustrated in figure 3.1 with ferulic acid as the reducing substrate.

$$E(Fe^{III}, R) + H_2O_2 \xrightarrow{k_1} \underset{\text{Compound I}}{E(Fe^{IV}{=}O, R^{\bullet+})} + H_2O \quad (2)$$

$$E(Fe^{IV}{=}O, R^{\bullet+}) + AH_2 \xrightarrow{k_2} \underset{\text{Compound II}}{E(Fe^{IV}{=}O, R)} + AH^{\bullet} \quad (3)$$

$$E(Fe^{IV}{=}O, R) + AH_2 \xrightarrow{k_3} E(Fe^{III}, R) + AH^{\bullet} + H_2O \quad (4)$$

$$2AH^{\bullet} \rightarrow A_2H_2 \quad (5)$$

E	= Enzyme
R	= site of cation radical
AH_2	= reducing substrate
$AH^{\bullet}$	= one-electron oxidation product of AH_2
k_1	= rate constant for compound I formation
k_2	= rate constant for compound I reduction
k_3	= rate constant for compound II reduction

The first step of the catalytic cycle, equation 2, can be defined as a two-electron transfer because no free radical substrate-intermediate is liberated from the enzyme during this reaction. In equation 2 H_2O_2 and the Fe(III) resting state of the enzyme react to yield compound I. Compound I, a high oxidation state intermediate, consists of a porphyrin-based cation radical and an Fe(IV) oxoferryl centre [62]. Compound I is two oxidizing equivalents above the resting state [4]. These oxidizing equivalents are retained in compound I as a porphyrin π-cation radical and a low-spin ferryl ion. Studies have shown that the dissociation constant of compound I is so low that its formation can be regarded as an irreversible process [3].

The first one-electron reducing step involves the reduction of compound I and subsequently generates compound II (equation 3). Compound II is a low-spin Fe(IV) oxoferryl species that is one oxidizing equivalent above the resting state. Both compound I and II are powerful oxidants with redox potentials of approximately + 1 V. The second one-electron reduction step restores compound II to the resting state of the enzyme (equation 4) [4]. Kinetic and equilibrium data show that both one-electron reducing steps are reversible [3]. The formation of compound III, a resonance hybrid of iron(III)-superoxide and iron(II)-dioxygen complexes, has also been reported [63].

HRP is susceptible to inactivation by peroxides when reducing substrates are absent and excess peroxide reacts with compound I. The nature of the peroxide itself determines the final form of the compound I-peroxide complex. In the case of H_2O_2 the complex can follow one of three possible pathways. The two catalytic pathways either return the complex to the resting state enzyme with the production of molecular oxygen or produces compound II, compound III and a superoxide radical anion. The competing inactivation pathway produces an inactive verdohemeperoxidase P-670 [64].

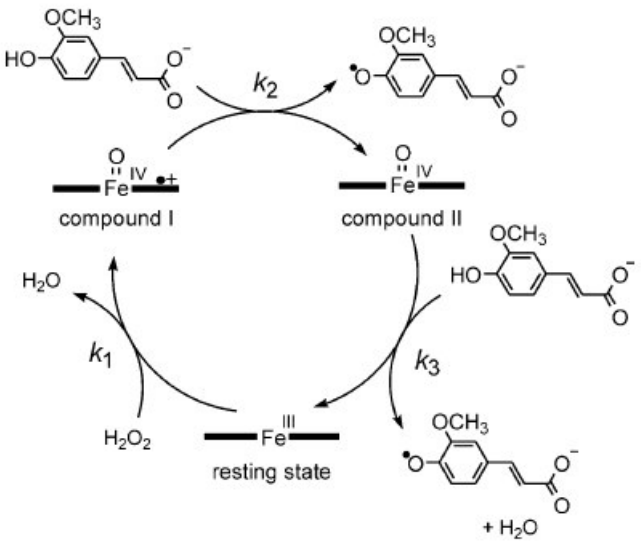

Figure 3.1. Catalytic cycle of HRP-C. *Reducing substrate*; ferulate. k_1; rate of compound I formation. k_2; rate of compound I reduction. k_3; rate of compound II reduction [4].

3.2.1 Ligand binding sites

HRP ligands are divided into two groups based on whether they bind to the sixth coordination site of the heme group or not. Small molecules such as azide, carbon monoxide and cyanide are only able to bind directly to the sixth coordination site in their protonated form. Arg38 and His42 stabilize six-coordinate peroxidase complexes in the distal heme pocket (see chapter 2.2.1 and chapter 2.6) [4].

3.2.2 Aromatic substrate binding sites

Enzyme inactivation studies have been used to explore the site of substrate oxidation [65-69]. It appears that substrate oxidation occurs at the "exposed" heme edge, a region containing the heme C18 methyl and heme C20 meso protons (see figure 2.5). The local protein environment appears to obstruct substrate access to the oxoferryl centre of HRP by means of a "protein-imposed barrier" [1]. This 'closed' heme structure explains why peroxidases are much less effective catalysts of oxygen-transfer reactions compared to cytochrome P450. Comparable studies show that modifications occur to the heme iron and pyrrole nitrogens of cytochrome P450. Site-directed mutagenesis of HRP has been used to increase accessibilty to the oxoferryl center of compound I by substituting Phe41 and His42 with smaller amino acids [70,71].

As mentioned in chapter 2.3.1, resting state HRP and some of its ligand-bound derivatives form stable, reversible 1:1 complexes with a variety of aromatic molecules in the absence of H_2O_2. Crystallographic studies have revealed the site where these aromatic substrates are located. Figure 3.2 illustrates the binding of ferulic acid to cyanide-ligated HRP. The aromatic ring of ferulic acid is located close to the exposed heme edge and the phenolic acid side chain is sloping towards the entrance of the binding site. Hydrogen-bonded interactions occur between Arg38 and ferulic acid and between ferulic acid and an active water molecule [4].

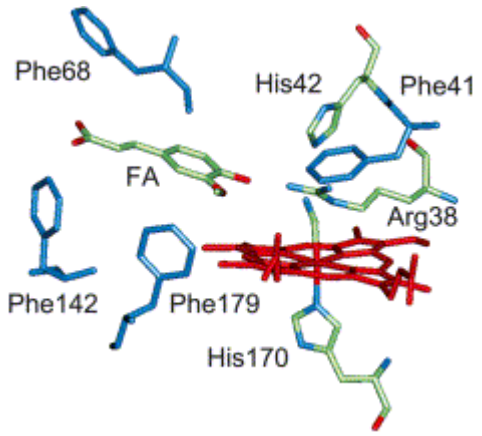

Figure 3.2. Binding of ferulic acid to cyanide-ligated HRP represented by X-ray crystallography (Brookhaven accession code 7ATJ). *Red*; heme group. *Blue*; phenylalanine side-chains contributing to the binding site. *Atom colours*; ferulic acid, cyanide ligand (bound to heme iron at vacant distal coordination site) and catalytic residues Arg38, His42 and His170 [4].

3.3 Functional roles of HRP in horseradish peroxidase

As mentioned in chapter 2, horseradish contains a collection of peroxidase isoenzymes that can potentially carry out a variety of functions. It is not yet known whether isoenzymes have specific functions in the plant, therefore the following comments apply to the enzyme in general. For the purpose of this discussion, the reactions catalyzed by HRP are simplified to equation 6:

$$H_2O_2 + 2AH_2 \xrightarrow{HRP} 2H_2O + 2AH^{\bullet} \qquad (6)$$

AH_2 = reducing substrate
$AH^{\bullet}$ = radical product

From this equation, two possible catalytic functions can be assigned to HRP: the conversion of H_20_2 to water and/or the oxidation of a substrate molecule to its free radical product [1]. Although HRP could theoretically protect the plant from peroxide, the conversion of H_20_2 to water is not the primary function of class III plant peroxidases such as HRP. Plants use other enzymes, including ascorbate peroxidase, to regulate intracellular levels of H_20_2. Therefore it is more likely that the radical products formed by HRP are indicators of possible *in vivo* functions [4].

HRP has been credited with a central role in numerous *in vivo* processes ranging from host defense mechanisms to hormone metabolism. These processes include resistance to infection by lignification and suberin formation as well as indole-3-acetic acid (IAA) metabolism [72]. External factors such as infection or wounding of plant tissue cause the expression of peroxidases involved in cross-linking reactions. Cross-linking reactions include the formation of dityrosine linkages [73], the formation of diferulate linkages from pectins or polysaccharides [74], the biosynthesis of lignin by oxidative coupling of phenolic compounds and the cross-linking of phenolic monomers in the formation of suberin [4]. Lignin is a complex, highly branched phenylpropanoid polymer that constitutes between 20-30% of cell walls [75]. Suberin is a polymeric lignin-like material that consists of phenylpropanoid, esterified fatty acid and alcohol components. When the plant is wounded, cross-linking reactions are initiated to form protective polymeric barriers of lignin and suberin. These barriers limit water loss and invasion by pathogens [1].

Plant peroxidases are known to degrade the plant growth hormone IAA. IAA metabolism is initiated when a trace of the IAA cation radical is produced. HRP is able to metabolize IAA in the absence of H_20_2 to produce 3-methylene oxindole, indole-3-methanol and indole-3-aldehyde. This complex mechanism of action has been studied in detail and has been used in the development of new cancer therapies (see chapter 4.4.10) [63, 76]. The physiological importance of IAA metabolism by HRP is still unclear: although increased HRP activity favours defensive strategies such as resistance to pathogens, the increase in IAA metabolism by HRP has a negative impact on plant growth [4]. The expression of HRP in plant tissue at different developmental stages must therefore reflect a balance between growth and defense [2,77].

Although many possible functions have been assigned to HRP such wide reactivity is not readily reconcilable with closely controlled processes such as hormone metabolism. The wide variety of the suggested functions is only possible if the expression of a specified activity is limited by the localization or unique properties of different isoenzymes [72]. Further characterization of isoenzyme expression and activity will help determine the specific roles of HRP *in vivo*.

3.4 Commercial applications of HRP

Horseradish is cultivated mainly for the culinary value of its roots. The commercial value of the peroxidase enzyme is, however, also an important addition to the economic importance of this plant. HRP, and more predominantly HRP-C, is isolated from horseradish roots on a relatively large scale for use in analytical, industrial and clinical applications. Other uses of the enzyme include organic synthesis, biotransformation and the treatment of waste water [4].

HRP is frequently used in immunoassays because of its high turnover rate and its stability upon storage. The enzyme can also withstand variations in temperatures, pH, ionic strength and buffer types. HRP-antibody conjugates for use in these immunoassays can easily be prepared by simple enzyme oxidation procedures. Highly specific and sensitive assays using colorimetric, fluorimetric or luminescent procedures to detect HRP activity have been designed. The enzyme-linked immunosorbent assay (ELISA) is an example of an immunoassay which uses colorimetric substrates of HRP as a rapid and convenient detection method [78].

Immunohistochemical and immunoblotting procedures also employ HRP where HRP-labeled reagents are detected using soluble chromogenic substrates which precipitate as a result of the enzymatic reaction. These substrates, when precipitated, leave an insoluble coloured product at the site of the bound enzyme. These procedures are used to locate and visualize functional locations of molecules *in vivo*. Additionally, HRP can be used as a non-isotopic label in DNA probes.

HRP is often incorporated in bi-enzyme systems (figure 3.3). Bi-enzyme systems are included in bioassays where a detectable product is not directly produced. HRP is then used to convert the H_20_2 product of an oxidase reaction to a detectable form. Bi-enzyme systems are often immobilized onto electrodes for use in biosensors. A biosensor, comprising of an immobilized bio-component (HRP) in close proximity of a transducing element, is designed to convert biochemical responses to electrical responses. A biosensor has two transducers which relate the concentration of an analyte to a measurable signal. The analyte is firstly converted into a chemical and/or physical response by the biochemical transducer and this response is then converted into an electrical signal by the physical transducer. Biosensors using HRP have been developed for the quantification of alcohol, choline and cholesterol [79].

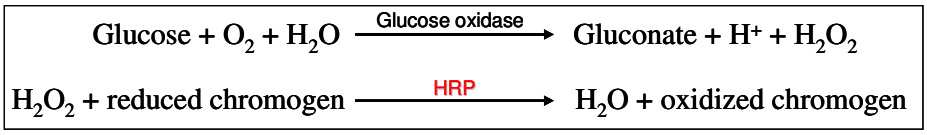

Figure 3.3. Bi-enzyme system involving HRP. The glucose oxidase reaction produces a substrate (H_2O_2) for HRP, which in turn oxidizes the chromogen and allows quantification of glucose [78].

3.4.1 Targeted cancer therapy

Recent studies have proposed the use of IAA metabolism by HRP as a basis for a novel targeted cancer therapy [80]. IAA shows no adverse side-effects in humans and is not easily oxidized by mammalian peroxidase [80-83]. IAA metabolites, specifically 3-methylene-2-oxindole, show high reactivity towards cellular nucleophiles and are therefore toxic towards mammalian cells, including tumour cells [84]. The challenge is to develop this system for *in vivo* implementation so that the toxic IAA metabolites are only produced in the tumour and do not damage normal tissue. HRP-C is ideal for the development of antibody-, gene- or polymer-directed enzyme:prodrug therapies due to the enzyme's stability at 37ºC and high activity at physiological pH. Also, HRP shows no toxicity towards mammalian cells and can easily be conjugated to antibodies [4].

The development of potential enzyme:prodrug therapies such as targeted cancer therapy is clearly dependant on a comprehensive understanding of the enzyme's physicochemical and kinetic properties. The successful production of recombinant HRP and the subsequent characterization of the enzyme have led to major advances in clinical applications of HRP. A brief overview of recombinant HRP is therefore given in chapter 4.

4. RECOMBINANT HORSERADISH PEROXIDASE

4.1 Introduction

Detailed reviews on the history and development of peroxidase are available in the literature [63, 85, 86]**.** Briefly, the first record of an HRP-catalyzed reaction was published in 1810 when Louis Antoine Planche reported the development of a brilliant blue colour when a fresh piece of horseradish root tissue (figure 4.1) was soaked in a tincture of guaiacum (powdered heartwood from *Guaiacum officinale* and *G. sanctum* containing various phenolic compounds). The observed colour reaction was most likely the oxidation of 2,5-di-(4-hydroxy-3-methoxyphenyl)-3,4-dimethylfuran by the peroxidase to the corresponding bis-methylenequinone, also known as guaiacum blue [87].

The term 'peroxidase', however, only came into use at the end of the nineteenth century. Although crude HRP extracts were obtained at the beginning of the twentieth century [88], it was only in 1942 that the enzyme was purified sufficiently for crystallization [46]. By 1950, research established heme and carbohydrate components for HRP. Further advances in the twentieth century include the first kinetic analysis of H_2O_2 reduction by HRP as well as observations of catalytic intermediates, compound I and II. The amino acid sequence of horseradish peroxidase was determined in 1976 [5] and its three-dimensional structure solved in 1997 using recombinant HRP (figure 2.3) [13]. Recombinant HRP, produced in *Escherichia coli*, is non-glycosylated and is therefore ideal for use in crystallographic analysis. Additionally, recombinant HRP has allowed the high resolution description of the catalytic intermediates of the enzyme [4,28].

The successful production of recombinant HRP-C (HRP-C*) has led to major advances in our knowledge of the enzyme's structure and function. Continuous research through site-directed mutagenesis and directed-evolution techniques using HRP-C* has made a significant contribution to our understanding of peroxidase enzymes in general.

A  **B** 

Figure 4.1. *A.* The horseradish, *Amoracia rusticana*. *B*. Horseradish root.

4.2 Amino acid sequence and genes of HRP

The complete amino acid sequence of HRP-C was published in 1976 and is discussed in detail in chapter 2.2. The amino acid sequences that were subsequently determined for HRP-A2 and HRP-E5 show only 54% and 70% identity when compared to the sequence of HRP-C. This suggests that distinct peroxidase isoenzymes are present in horseradish roots. As mentioned in chapter 2.7, it is still unclear which HRP-isoenzymes are encoded for by specific genes and which isoenzymes are produced by post-translational modifications [18].

Since the identification of at least 28 different peroxidase genes in *Arabidopsis thaliana*, estimates for the number of peroxidase genes in *A. rusticana* has increased significantly [51-54]. Four genomic DNAs encoding for neutral HRP-isoenzymes have been cloned and characterized from *A. rusticana* [89]. Northern blot analysis has been used to examine the organ-specific expression of these peroxidase-encoding genes. Two of these genes (*prx*C1*a* and *prx*C1*b*) are expressed in roots and stems while *prx*C2 and *prx*C3 are mainly expressed in roots. All four genes are located in tandem on the chromosome and consist of three introns and four exons. The genes also have identical splice site positions, a common trend in other plant peroxidase genes which could be of evolutionary importance [30,31].

Three cDNAs (*prx*C1*a*, *prx*C1*b* and *prx*C1*c*) have also been isolated from cultured cells of *A. rusticana* [90]. These cDNAs are highly homologous (91 – 94%) and all three contain a C-terminal extension of 15 amino acid residues plus a hydrophobic leader sequence of 30 amino acids [91]. It is speculated that the C-terminal extension is required for vacuolar targeting [90]. With the exception of the hydrophobic leader sequence and C-terminal extension, the amino acid sequence determined from *prx*C1*a* is identical to the amino acid sequence of HRP-C. The transcriptional regulation of *prx*C2 has been studied extensively [70]. It is well-known that wounding of horseradish leaves induces peroxidase activity and studies have shown that only the mRNA for *prx*C2 accumulates after wounding [92].

The only other HRP cDNA to be cloned and sequenced corresponds to a neutral isoenzyme, HRP-N. Although an active form of HRP-N can be produced using recombinant technology this isoenzyme has yet to be isolated from horseradish roots. Genes that encode other HRP-isoenzymes have not yet been cloned but counterparts have been identified in *A. thaliana* [93].

4.3 Expression of recombinant HRP

Three synthetic HRP-C genes, based on the amino acid sequence of HRP-C, have been synthesized in order to successfully express HRP-C* [28, 94, 95]. Numerous expressions systems for HRP-C*, including *baculovirus*, *E. coli,* and *Saccharomyces cerevisiae* have been evaluated [96-101].

The *baculovirus* system can be used to produce highly glycosylated, active HRP-C* in yields of 5-10 mg/ℓ [96]. Conversely, the *E. coli* system produces non-glycosylated apo-HRP* in yields of 2-4 mg/ℓ [102, 103] and *S. cerevisiae* systems show low expression levels and low yields of hyper-glycosylated enzyme (50 µg/ℓ) [100]. The *E. coli* system produces lower yields of HRP* because the enzyme must be recovered from inclusion bodies after expression [29]. This recovery process involves the solubilization of the inclusion bodies, the controlled reoxidation of the reduced denatured enzyme and *in vitro* refolding in the presence of calcium and heme [4]. Although the refolding of HRP-C* recovered from inclusion bodies is a relatively low-cost method, it is also rather inefficient. One advantage of the *E. coli* system is the expression of an active non-glycosylated enzyme which can be used in crystallographic studies. Both *E. coli* and the *baculovirus* systems have been used to produce site-directed mutants. These mutants are used to identify essential amino acids, characterize binding sites and study the effects of mutations on catalytic activity [1].

Directed-evolution techniques have been used in attempts to improve qualities of HRP-C for diagnostic and biotechnological applications. For these studies it is advantageous to express HRP-C* in a *S. cerevisiae* system rather than an *E. coli* system so as to produce an active enzyme without the need for *in vitro* refolding. Directed-evolution using random point mutagenesis and screening has been able to improve the activity, thermal stability and resistance to inactivation of HRP-C*. A recombinant enzyme exhibiting a 3-fold improvement in thermal stability at 60 ºC and pH 7.0 was produced by combining a HRP-C mutant evolved for higher activity with a single site mutation at Asn175 [104].

4.4 The plant peroxidase superfamily

Peroxidases have been isolated and characterized from many different living sources. Most peroxidases incorporate a prosthetic group usually based on ferriprotoporphyrin IX, although additional classes containing either selenium or vanadium have also been identified [10-107]. The heme-containing peroxidases are divided into the mammalian peroxidase superfamily and the plant peroxidase superfamily.

The plant peroxidase superfamily consists of bacterial, fungal and plant peroxidases that are divided into three classes: class I (yeast cytochrome *c* peroxidase, ascorbate peroxidases and gene-duplicated bacterial peroxidases), class II (fungal peroxidases) and class III ("classical" secretory plant peroxidases). These classes were primarily based on amino acid sequence comparisons but crystallographic studies have shown that the structural topology is in fact more conserved among the three classes than amino acid sequence identity [1]. Class I peroxidases do not contain disulphide bridges, unlike class II or III peroxidases. All HRP-isoenzymes contain ferriprotoporphyrin IX and are class III peroxidases. HRP and other class III peroxidases contain a 'core' peroxidase fold, which is conserved among all three classes of peroxidase and three additional α-helices [4].

Complementary data for other class peroxidases are often available and therefore a comparison with class I and class II peroxidases has proven useful in structure:function studies of HRP [18]. Investigations into the three-dimensional structure of HRP were preceded by studies on other peroxidases. By 1980 cytochrome *c* (class I peroxidase) was the first heme-containing peroxidase to be solved by crystallography [108]. The X-ray crystal structure of cytochrome *c* was subsequently refined and allowed the first comparisons between this enzyme and HRP based on primary and tertiary structure [109]. The similarity between the heme-linked regions and hydrophobicity profiles of cytochrome *c* and HRP suggested the two enzymes share a similar fold in spite of only 20% sequence identity [110]. These comparisons lead to low-resolution modelling of core α-helices of HRP as well as deductions of helical connectivities [109].

In contrast to class II fungal and class III plant peroxidases, cytochrome *c* contains no calcium binding sites; therefore solution of the crystal structures of class II peroxidases [111, 112] allowed more detailed homology modelling investigations of HRP. Structurally derived sequence alignments were later used to identify 10 structurally conserved regions. A full protein model of

HRP was sequentially built on a core peroxidase fold constructed from the coordinates of the 10 conserved regions [113]. This structure predicted the heme-linked regions and calcium sites with reasonable accuracy but failed to successfully predict a large untemplated insertion containing two additional α-helices unique to plant peroxidases [113, 114]. By 1995 both crystals and heavy atom derivatives for recombinant HRP were obtained [115]. This data, along with the first crystal structure of a native class III plant peroxidase (peanut peroxidase) led to a molecular replacement solution for recombinant HRP [13].

4.4.1 Arabidopsis thaliana *peroxidases*

A. thaliana has been named the 'model' plant and is also a member of the *Cruciferae* family. The results of a systematic survey of *A. thaliana* indicate that a single plant species contain many true peroxidase isoenzymes [1]. Sequencing of the genome of *A. thaliana* has provided the opportunity to investigate a whole family of peroxidases from a plant. The peroxidase gene family of *A. thaliana* has also allowed comprehensive investigations into evolutionary relationships between class III peroxidases from different plant species. It has been suggested that the class III plant peroxidase gene family developed when plants colonised the land and peroxidase functionality presented adaptive advantages. These advantages include defence against pathogens and cell-wall metabolism [116-118]. Evolutionary studies of peroxidases suggest that some groups of peroxidases evolved independently in eudicots and monocots while others were lost. This implies that each plant species has a relatively large number of distinctive peroxidase isoenzymes [4].

The peroxidase gene family of *A. thaliana* contains approximately 73 full-length genes of which 71 are predicted to code for stable enzymes that fold similarly to HRP-C [118]. Amino acid sequences of these predicted peroxidases have been used to identify potentially similar enzymes in other plant species. For example, an acidic peroxidase isoenzyme from *A. thaliana* (AtP-A2) shows 95% sequence identity with HRP-A2. Although the functional role of HRP-A2 is unknown, expression of AtP-A2 coincided with lignification and therefore offers a clue to the *in vivo* function of HRP-A2 [119]. Also, the three-dimensional structure of recombinant AtP-A2 has been solved and provides an excellent model for HRP-A2, which has not yet produced crystals suitable for crystallographic studies [55].

As mentioned in the introduction to this chapter, relationship studies such as comparisons with *A. thaliana* have revealed possible functions for HRP and provide valuable insights into the enzyme's molecular properties and catalytic mechanism. These insights have contributed significantly to the commercial production and application of HRP which form the basis of the experimental work to be discussed in the following chapters.

5. ISOLATION AND PURIFICATION OF ISOENZYMES

5.1 Introduction

Various techniques have been developed to isolate HRP from horseradish roots and advances in the purification of HRP have been well documented [85, 86]. Although new techniques using aqueous two-phase partition systems [120] or reversed micelles [121, 122] have been investigated, most industrial purification methods are based on a series of chromatographic steps [1].

HRP is generally extracted from the crude plant material by homogenization and filtration, followed by salt fractionation of the primary root extract. Different chromatographic steps are then applied to further purify the enzyme. Purification methods using ion-exchange, size-exclusion or affinity chromatography exclusively or a combination of these methods are common practice. HRP-isoenzymes differ significantly in terms of pI and therefore ion-exchange chromatography is more frequently used for industrial purification of HRP and the systematic isolation of isoenzymes [50]. A single ion-exchange chromatography step is not, however, always sufficient and re-chromatography of the various isoenzymes may lead to protein loss. Additionally, partial irreversible protein adsorptions may lead to low purification yields [72].

These disadvantages are less prominent in affinity chromatography and therefore the use of affinity chromatography in HRP purification has been suggested as an improved purification method. Affinity chromatography matrixes with either an agarose derivative-bound aromatic hydroxamic acid or Sepharose-bound concanavalin A (Con A) have been developed to isolate HRP-isoenzymes. The ‘hydroxamate’ method exploits the differential affinity of the isoenzymes for the aromatic hydroxamic acid. The aromatic hydroxamic acid shows preferential affinity for the substrate binding sites of HRP, particularly for HRP-C. This method can reportedly separate HRP-C from acidic isoenzymes and other components in root extracts in a single step, replacing three or four stages of ion-exchange chromatography. Sepharose-bound Con A affinity matrixes have been developed for HRP purification but are generally less affective than the hydroxamate method. Also, the Sepharose-bound Con A method does not lend itself to resolution of HRP isoenzymes [72].

Despite the advantages of affinity chromatography in HRP purification, ion-exchange chromatography is a decidedly faster method for peroxidase purification. Additionally, in the case of the hydroxamate method, the affinity ligand is slowly degraded by some plant components and

therefore ion-exchange chromatography needs to be included as a pre-purification step [72]. It is apparent that combinations of ion-exchange and affinity chromatography should be investigated for optimal HRP purification in future.

In this study, a current industrial method using ammonium sulphate fractionation and cation-exchange chromatography was adapted to purify crude HRP from horseradish roots. Apo-HRP was subsequently prepared from crude HRP by adapting industrial methods to lab scale. Cation-exchange chromatography was used to isolate HRP-isoenzymes from crude HRP.

5.2 Isolation and purification of crude HRP from horseradish roots

Figure 5.1 is a schematic representation of the procedure used in this study to isolate crude HRP from horseradish roots as adapted from current industrial methods.

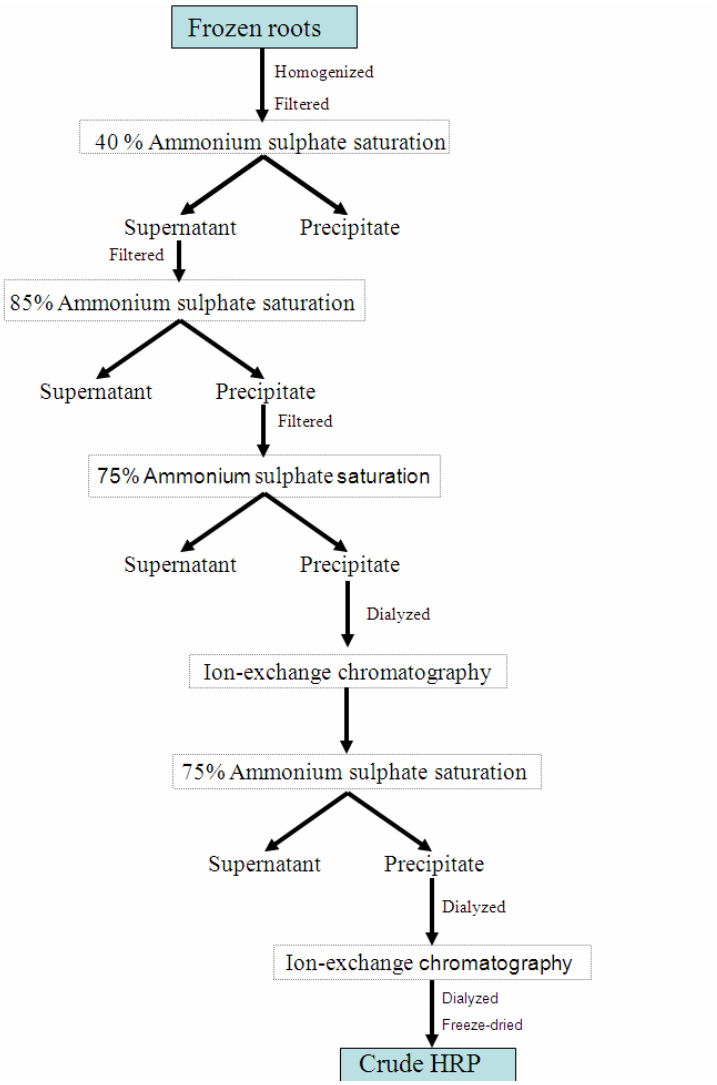

Figure 5.1. Schematic representation of the isolation of crude HRP from horseradish roots.

Frozen roots were homogenized in three volumes of 0.4 M NaCl. For the purpose of this study, the homogenate will be referred to as primary root extract. The primary root extract was filtered through 100 micron nylon mesh, the solid waste discarded and the resultant filtrate clarified through a Hyflo filter-aid bed on filter paper under negative pressure. Solid ammonium sulphate was added to the filtrate (industrially known as the 'clarified' extract) to 40% saturation and the pH adjusted to 5.0. All ammonium sulphate fractionation steps were carried out at 20˚C. The precipitate was allowed to settle before the supernatant was clarified through a Hyflo filter-aid bed and brought to 85% ammonium sulphate saturation. The solution was again filtered through a Hyflo filter-aid bed. The precipitate, together with the filter-aid, was collected from the filter and dissolved in a minimal volume of deionised water. This solution was filtered through filter paper and washed with deionised water until the wash was clear. The filtrate was subsequently brought to 75% ammonium sulphate saturation and precipitation of the protein was allowed to take place. The precipitate was collected by filtration through Viking filter paper and dissolved in a minimal volume of deionised water. The dissolved precipitate was dialyzed overnight against 20 ℓ deionised water.

The dialysate was transferred to a carboxymethyl (CM)-Sepharose column (3 x 27 cm) previously equilibrated with 5 mM sodium acetate (pH 4.5). The resin was washed with one volume of equilibration buffer before eluting with 50 mM sodium acetate (pH 4.5) at a flow rate of 2 ml/min. Absorbance was measured at 280 nm and fractions containing HRP were identified from the elution profile after HRP activity assays (figure 5.2). These fractions were combined, brought to 75% ammonium sulphate saturation and allowed to precipitate overnight. The precipitate was dissolved in deionised water and dialyzed overnight. The dialysate was transferred to a CM-Sepharose column (1 x 5 cm) previously equilibrated with 5 mM sodium acetate (pH 4.5). The resin was washed with one volume of equilibration buffer before eluting with 50 mM sodium acetate (pH 4.5) at a flow rate of 1 ml/min. Fractions containing HRP were collected and combined (figure 5.3).

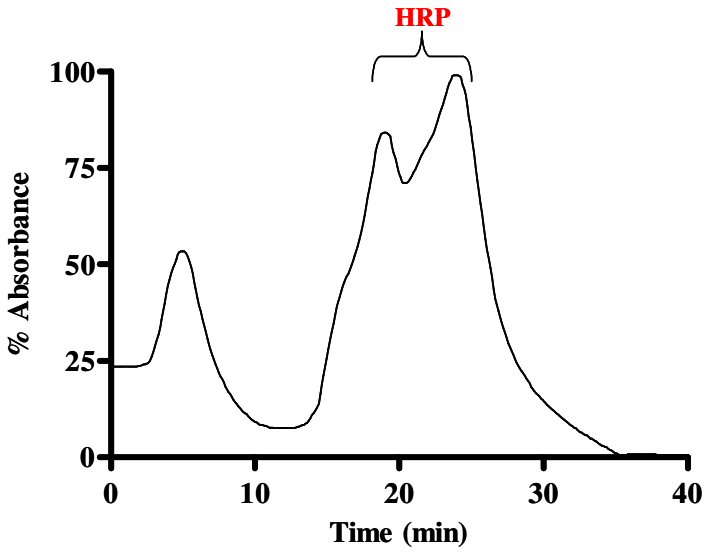

Figure 5.2. Elution profile of HRP from a CM-Sepharose column (3 x 27 cm) equilibrated with 5 mM sodium acetate (pH 4.5). *Elution buffer*; 50 mM sodium acetate (pH 4.5). Absorbance was detected at 280 nm.

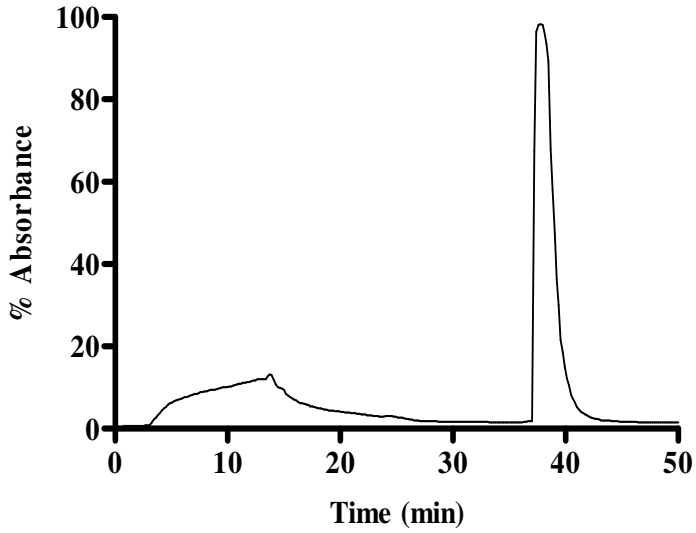

Figure 5.3. Elution profile of HRP from a CM-Sepharose column (1 x 5 cm) equilibrated with 5 mM sodium acetate (pH 4.5). *Elution buffer*; 50 mM sodium acetate (pH 4.5). Absorbance was detected at 280 nm.

The protein concentration of certain fractions obtained from the purification of HRP is given in table 5.1. Peroxidase activity was measured according to the method of Bergmeyer [123] using guaiacol as hydrogen donor. The enzymatic activity was determined by the rate of H_2O_2 decomposition by HRP, measured spectrophotometrically as the rate of colour development at 436 nm. One unit of enzyme activity (U) is equal to an absorbance change of 0.001 per minute at 25°C and pH 7.0. Specific activity for commercial HRP is approximately 300 U/mg.

The *Reinheitszahl* (RZ) for the fractions is also included in table 5.1. RZ is commercially used as a convenient measure of peroxidase purity. This value is the ratio of the absorbances recorded at 403 nm and 280 nm using UV-VIS spectrophotometry. Proteins absorb at 280 nm while absorbance at 403 nm is specific for heme-containing peroxidase. Values ranging from 3.0 – 3.4 are commercially accepted for high-quality peroxidase preparations [1]. This absorbance ratio has been reported to vary between 2.50 and 4.19 for different isoenzymes [2]. RZ determination is influenced by buffer and pH and therefore the accuracy of this ratio, as a measure of purity, is questionable. In this study RZ was merely used as an indication of HRP-content of fractions.

Table 5.1. Stepwise purification of crude HRP from 1000 g of horseradish roots.

Sample	Volume (ml)	Total protein (mg)	Total activity* (U)	Yield (%)	Specific activity (U/mg)	°purity	RZ (A_{403}/A_{280})
Clarified extract	3 070	13 304.37	17 856.56	100.00	1.34	1	0.01
0-40% ammonium sulphate supernatant	3 320	9 321.67	16 732.80	93.71	1.80	1.34	0.06
40-85% ammonium sulphate precipitate (dialyzed)	116	1 445.57	10 106.08	56.60	6.99	5.21	0.09
First chromatographic fraction	55	47.56	7 593.88	45.38	159.67	118.96	1.35

* One unit of enzyme activity (U) is equal to an absorbance change of 0.001 per minute at 25°C and pH 7, [123]

5.2.1 Preparation of apo-HRP from crude HRP

Apo-HRP was prepared from a crude HRP preparation (provided by BBI Enzymes). For the purpose of this study, 'crude' HRP refers to HRP prepared from frozen roots by the method summarized in figure 5.1. Apo-HRP was prepared from this crude enzyme preparation through repeated acidified acetone precipitation steps and centrifugation. The resulting protein was dialyzed against deionised water and freeze-dried. Enzymatic assays showed no peroxidase activity for the apo-HRP prepared in this study (results not shown).

5.3 Isolation and purification of HRP-isoenzymes from crude HRP

HRP-isoenzymes were isolated and purified from a crude HRP preparation. A sample of crude HRP was dialyzed against deionised water and the dialysate subsequently filtered using hydrophylic polyvinylidene difluoride syringe-tip filters (0.45 μm pore size). The crude HRP sample was transferred to a CM-Sepharose column (3 x 27 cm) previously equilibrated with 5 mM sodium acetate (pH 4.4). The resin was washed with equilibration buffer until all non-adsorbed proteins had eluted as indicated by absorbance at 280 nm. The protein was then eluted from the column with a linear gradient consisting of equal volumes 5 mM sodium acetate (pH 4.4) and 50 mM sodium acetate (pH 4.4). A linear flow rate of 9.76 cm/h was maintained and the elution profile is shown in figure 5.4.

Absorbance was detected at 280 nm and fractions containing peak B were identified from the elution profile and combined. This combined fraction, fraction B, was dialyzed against deionised water overnight and freeze-dried. Fractions containing peak A were identified from the elution profile and combined. This combined fraction, fraction A, was transferred to a CM-Sepharose column (3 x 27 cm) previously equilibrated with 5 mM Tris-HCl (pH 8.0). The resin was washed with equilibration buffer until non-absorbed isoenzymes eluted as indicated by absorbance at 280 nm. The proteins were eluted from the column with 10 mM sodium carbonate (pH 9.6) and the eluent was dialyzed against deionised water overnight. After dialysis, the dialysate was freeze-dried. The protein concentration of the crude HRP sample and both isolated fractions is given in table 5.2. Specific activity and *RZ* was determined as previously described.

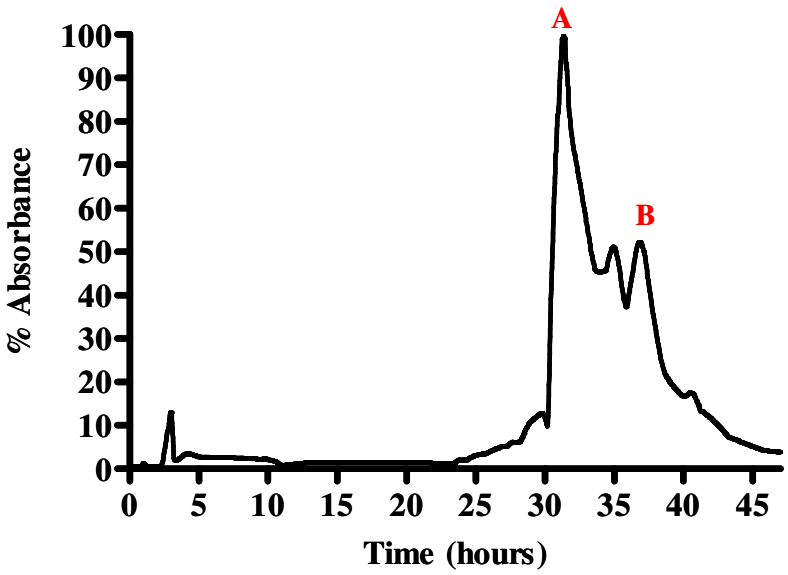

Figure 5.4. Elution profile of HRP from a CM-Sepharose column (3 x 27 cm) equilibrated with 5 mM sodium acetate (pH 4.4). A linear gradient consisting of equal volumes 5 mM sodium acetate (pH 4.4) and 50 mM sodium acetate (pH 4.4) was used for separation. Absorbance was detected at 280 nm.

Table 5.2. Purification of HRP isoenzymes from crude HRP.

Sample	Dry weight (mg)	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	°purity	RZ (A_{403}/A_{280})
Crude HRP	1 500	447.71	151 250.82	100.00	337.83	1.00	2.29
Fraction A*	276	90.25	9 921.18	6.56	109.93	0.33	2.95
Fraction B	192	86.01	10 165.52	6.72	118.19	0.35	2.6

*Protein determination and activity assay of dialyzed, freeze-dried fraction A

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify the purity and determine the approximate molecular mass of the isolated fractions. Figure 5.5 shows the electrophoretic analysis of a lysozyme standard, the isolated fractions and an HRP-C standard (provided by Roche) on a 12% polyacrylamide gel. Staining with Coomassie Brilliant Blue revealed homogenous migration for the HRP fractions as well as the HRP-C standard. A molecular mass (Mr) marker was also loaded on the gel. The R_f values of the proteins in the Mr marker were used to draw a graph of R_f versus log Mr. Approximate molecular mass of the isolated isoenzymes in fraction A and B was determined using the respective R_f-value of each isoenzyme (see chapter 10.9.5). The molecular mass of the HRP-C standard as well as fractions A and B purified in this study was approximately 45 785.

Liquid chromatography mass spectroscopy (LCMS) was used to further analyse the purity and accurately determine the molecular mass of fractions A and B as well as the HRP-C standard. According to figures 5.6 and 5.7 the molecular mass of the HRP-C standard (43 173.50) corresponds to that of Fraction A (43 178.00). The molecular mass determined for Fraction B by LCMS was 42 356.10 (figure 5.8).

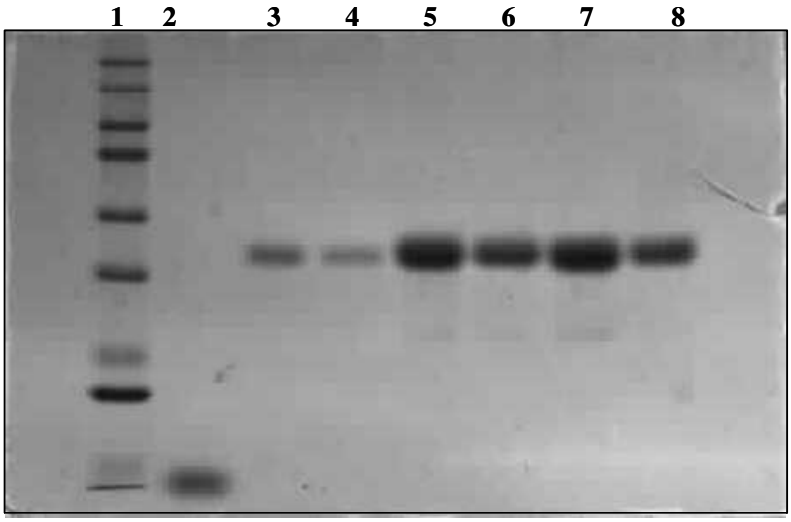

Figure 5.5. SDS-PAGE of isolated HRP-isoenzymes. Proteins were resolved under denaturing conditions on 12% poly-acrylamide gel. *Lane 1;* Mr marker (10-250 kDa). *Lane 2;* 4 µg lysozyme standard. *Lane 3;* 4 µg fraction B. *Lane 4;* 2 µg fraction B. *Lane 5;* 4 µg fraction C. *Lane 6;* 2 µg fraction C. *Lane 7;* 4 µg HRP-C standard. *Lane 8;* 2 µg HRP-C standard. Molecular masses of HRP-isoenzymes estimated in 37-50 kDa range.

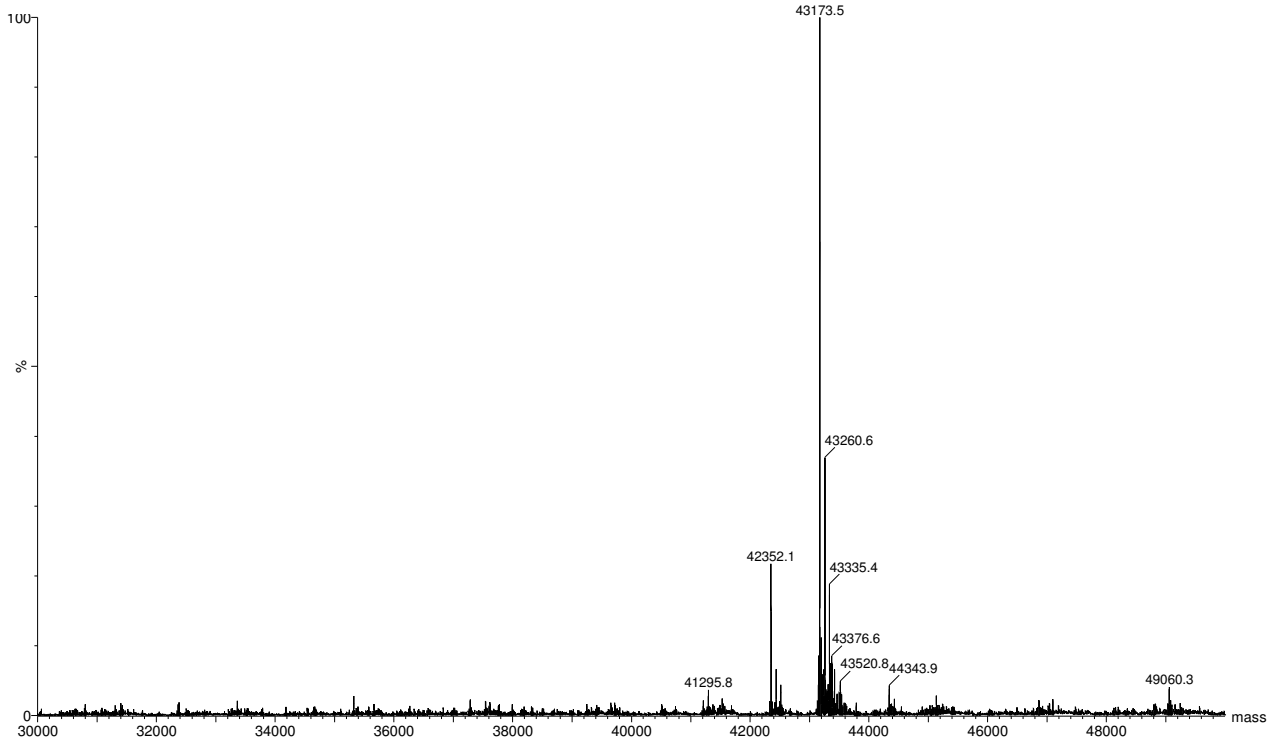

Figure 5.6. Full mass spectrum of HRP-C standard by LCMS analysis. Separation was carried out on a C_{18} column with an acetonitrile gradient containing 0.1% formic acid and detected by ESI-mass spectrometer in positive mode. The molecular mass of the HRP-C standard is 43 173.50.

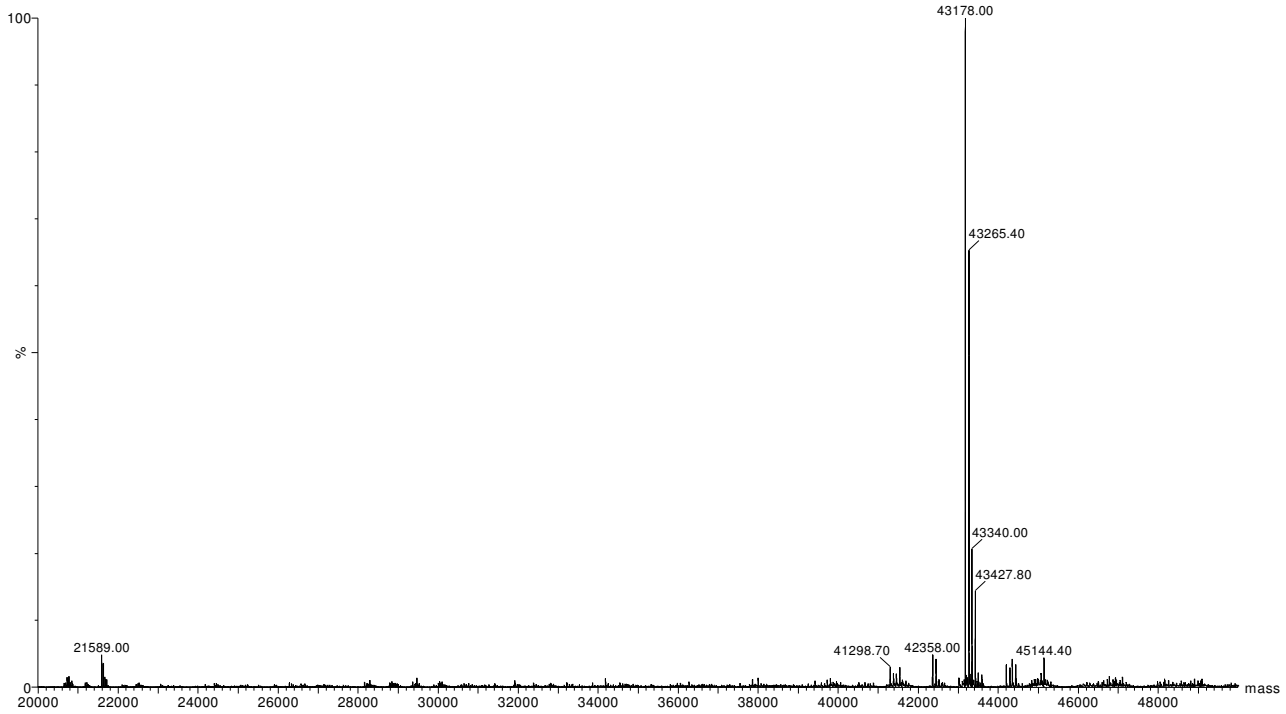

Figure 5.7. Full mass spectrum of fraction A by LCMS analysis. Separation was carried out on a C_{18} column with an acetonitrile gradient containing 0.1% formic acid and detected by ESI-mass spectrometer in positive mode. The molecular mass of fraction A is 43 178.0.

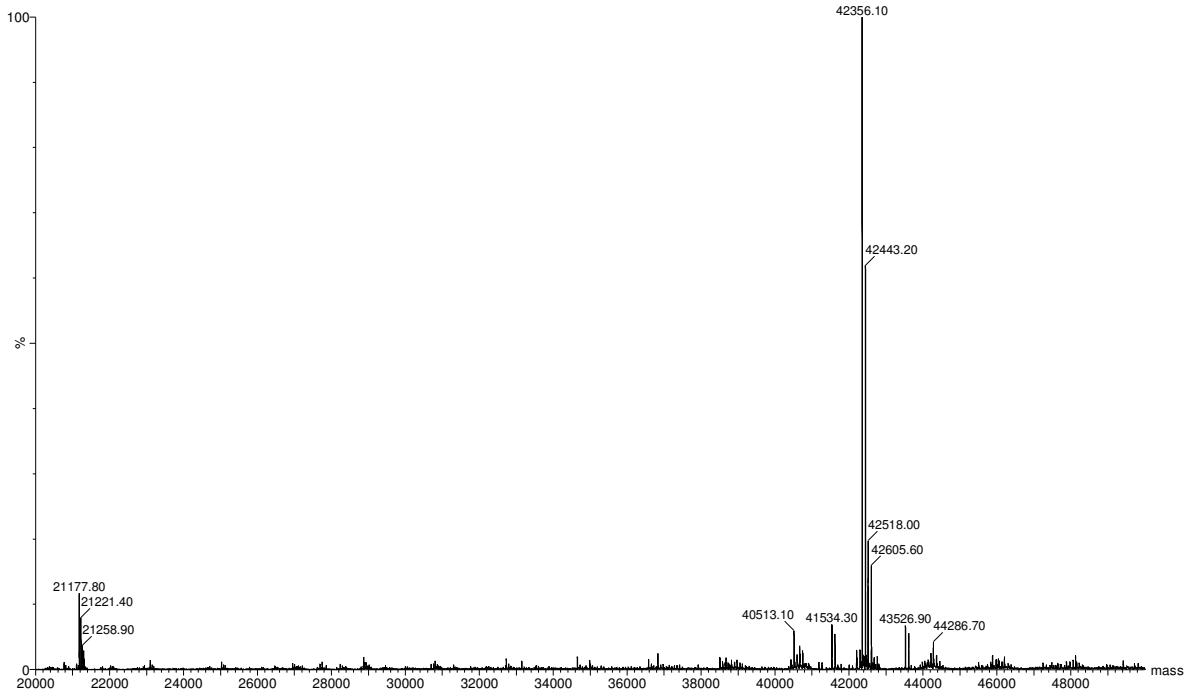

Figure 5.8. Full mass spectrum of fraction B by LCMS analysis. Separation was carried out on a C_{18} column with an acetonitrile gradient containing 0.1% formic acid and detected by ESI-mass spectrometer in positive mode. The molecular mass of fraction B is 42 356.10.

5.4 Discussion

In this study current industrial methods to prepare crude HRP, apo-HRP and HRP-isoenzymes were performed on laboratory scale. Crude HRP was isolated from horseradish roots using ammonium sulphate fractionation and cation-exchange chromatography. Although salt fractionation eliminates most of the contaminating proteins, protein determination and activity assays show that cation-exchange chromatography is essential to obtain enzyme of high purity (table 5.1). Apo-HRP was prepared from a crude HRP preparation through repeated acidified acetone precipitation steps. This procedure successfully removed heme from the enzyme, resulting in an apo-enzyme with no catalytic activity.

Cation-exchange chromatography was used to purify isoenzymes from a crude HRP sample. Table 5.2 shows significant losses in protein and activity. This study was not focused on isoenzyme production yield but rather was concerned with isolating HRP-C and another isoenzyme of

sufficient purity. Methods were adapted from current industrial methods but are not necessarily a true reflection of large scale production yields. RZ is commonly used in the industry as a measure of peroxidase purity. Although it is not a definitive indication of purity, RZ for HRP-C purified in this study does fall within the range that is commercially accepted as sufficient purity.

The purity and molecular mass of the isolated isoenzymes were more accurately determined by two other methods. Firstly, SDS-PAGE showed single bands for both isoenzymes isolated and the migration corresponded with that of the HRP-C standard. The molecular mass for fraction A, B and the standard as determined by SDS-PAGE was approximately 45 785.

LCMS also revealed that the fractions isolated in this study each contained a dominant form of HRP. Comparison of the full mass spectra of the HRP-C standard and fraction A as well as the molecular mass determined for the major component of both the HRP-C standard (43 173.5) and fraction A (43 178) showed that fraction A consisted of mainly HRP-C. The study will therefore refer to fraction A as HRP-C. The molecular mass of the major component of fraction B (42 356.1) was 822 less than that of HRP-C and this difference is approximately the equivalent of a carbohydrate moiety. Fraction B could therefore be a less glycosylated form of HRP-C or one of the HRP-B isoenzymes. For the purpose of this study the difference between fraction A and B was sufficient for immunological studies (see chapter 7) and, therefore, fraction B was not further analysed. Amino acid analysis would be necessary to further identify and characterize fraction B. Fraction B will be referred to as HRP-B in the following chapters.

6. CHROMATOGRAPHIC CHARACTERIZATION OF HRP

6.1 Introduction

HRP, specifically isoenzyme C, has many commercial applications aside from its biological function in the plant, as discussed in chapter 3. The HRP-C content of primary root extracts and the subsequently purified peroxidase determine the commercial value of a batch of processed roots. At present, manufacturers of peroxidase suffer substantial financial losses due to the inability to determine the HRP-C content of raw material or monitor HRP-C levels during purification.

In this the use of HPLC as a possible isoenzyme detection method due to the high resolving power of this technique was investigated. Presently, cation-exchange HPLC is used industrially to examine the content of an HRP sample at an advanced stage of purification. This established method was also used to determine the purity of HRP-C isolated in this study and to monitor HRP-content during the purification process.

In this study the use of reversed-phase HPLC as an HRP detection method was also investigated. It was expected that, if the various forms and components of HRP differed in hydrophobicity, the retention time for each would be specific and could be used to examine the content of raw material.

6.2 Cation-exchange HPLC

An established salt-gradient elution system and a CM ion-exchange column (Waters Protein PAK CM, 8-HR, 8 μm) were used to characterize HRP. A linear gradient consisting of eluent A (0.01 M sodium acetate, pH 4.4) and eluent B (0.01 M sodium acetate, 0.2 M NaCl, pH 4.4) at a flow rate of 0.8 ml/min was used during separations (table 6.1). Samples were dissolved in eluent A and absorbance was measured at 280 nm and 403 nm, specific for proteins and heme, respectively. An HRP-C standard (provided by Roche), HRP-C isolated in this study as well as crude HRP samples were analyzed in triplicate (figures 6.1 – 6.3). Reproducible chromatograms could not be obtained for HRP-B (results not shown).

Table 6.1. Gradient elution system used in cation-exchange HPLC analysis.

Time (min)	Eluent flow rate (ml/min)	% Eluent A[a]	% Eluent B[b]
1	0.8	97	3
22	0.8	67	33
25	0.8	0	100
30	0.8	0	100
35	0.8	97	3

[a] 0.01 M acetate buffer, pH 4.4
[b] 0.01 M acetate buffer, pH 4.4, 0.2 M NaCl, pH 4.4

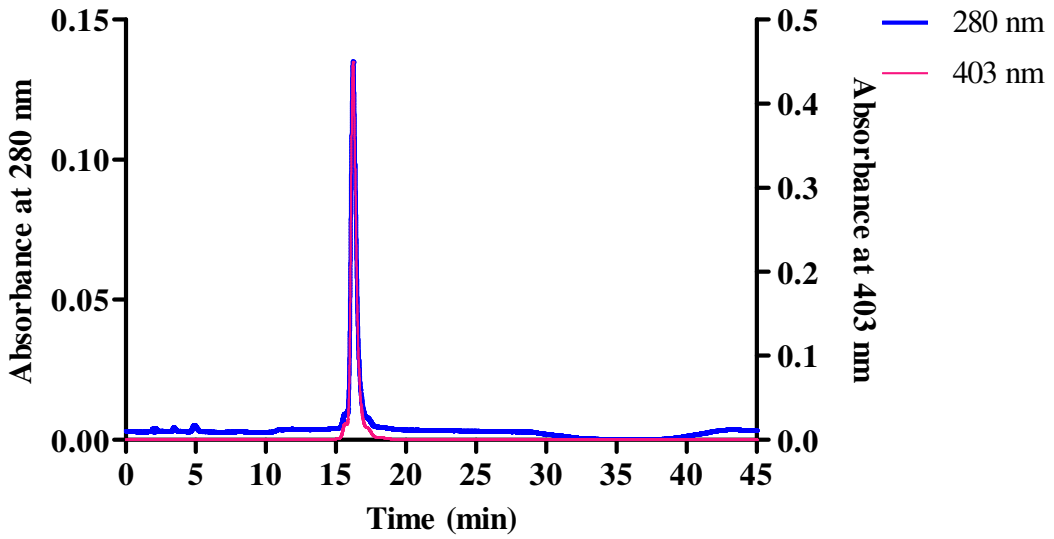

Figure 6.1. Elution profile of an HRP-C standard from a CM ion-exchange column. A linear gradient was run from 3% B to 100% B over 25 minutes. *Eluent A*; 0.01 M sodium acetate (pH 4.4). *Eluent B*; 0.01 M sodium acetate, 0.2 M NaCl (pH 4.4). *Sample injected*; 30 µl of 1 mg/ml HRP-C standard in eluent A. The HRP-C standard elutes at 16 minutes.

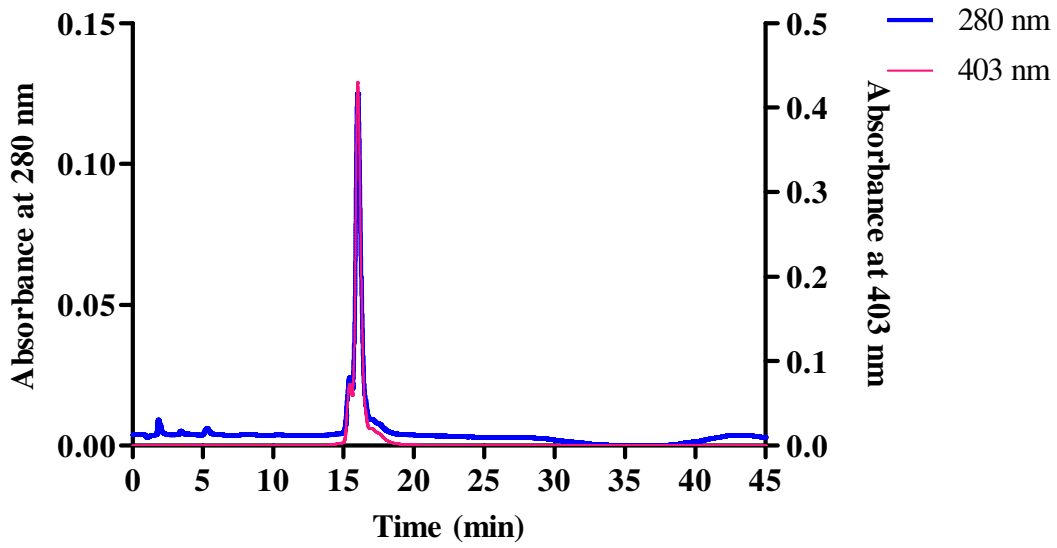

Figure 6.2. Elution profile of HRP-C from a CM ion-exchange column. A linear gradient was run from 3% B to 100% B over 25 minutes. *Eluent A*; 0.01 M sodium acetate (pH 4.4). *Eluent B*; 0.01 M sodium acetate, 0.2 M NaCl (pH 4.4). *Sample injected*; 30 µl of 1 mg/ml HRP-C in eluent A. HRP-C elutes at 16 minutes.

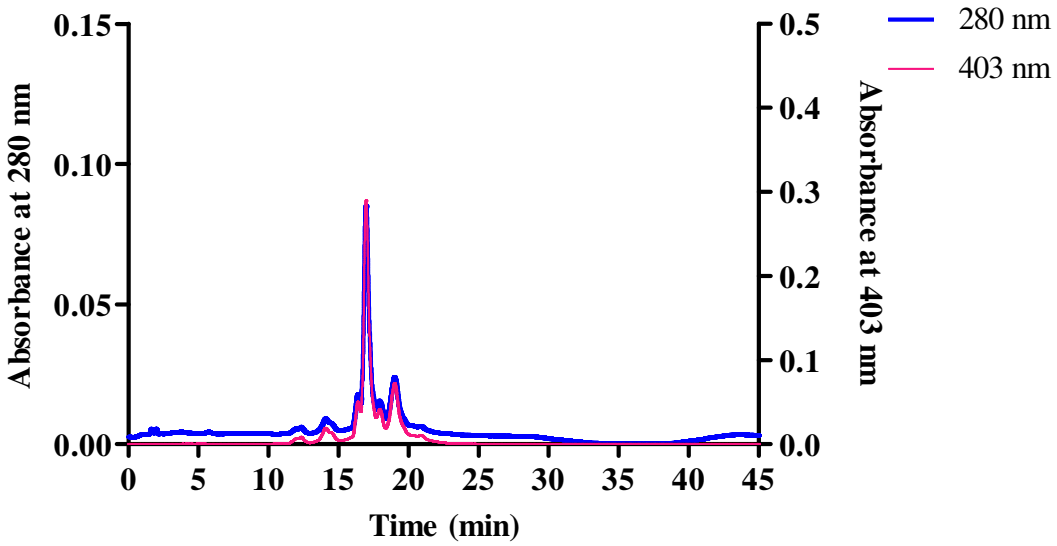

Figure 6.3. Elution profile of crude HRP from a CM ion-exchange column. A linear gradient was run from 3% B to 100% B over 25 minutes. *Eluent A;* 0.01 M sodium acetate (pH 4.4). *Eluent B*; 0.01 M sodium acetate, 0.2 M NaCl (pH 4.4). *Sample injected*; 30 µl of 1 mg/ml crude HRP in eluent A. HRP elutes at 16 and 18 minutes.

Samples from the stepwise purification of crude HRP from horseradish roots (chapter 5.2) were also analyzed (figures 6.4 and 6.5). Figure 6.4B shows the HPLC elution profile of the HRP-containing fraction (as annotated on figure 6.4A) collected during the first cationic chromatographic separation in the isolation of crude HRP (see figure 5.2). Similarly, figure 6.5B shows the HPLC elution profile of the HRP-containing fraction collected during the second chromatographic separation (see figure 5.3).

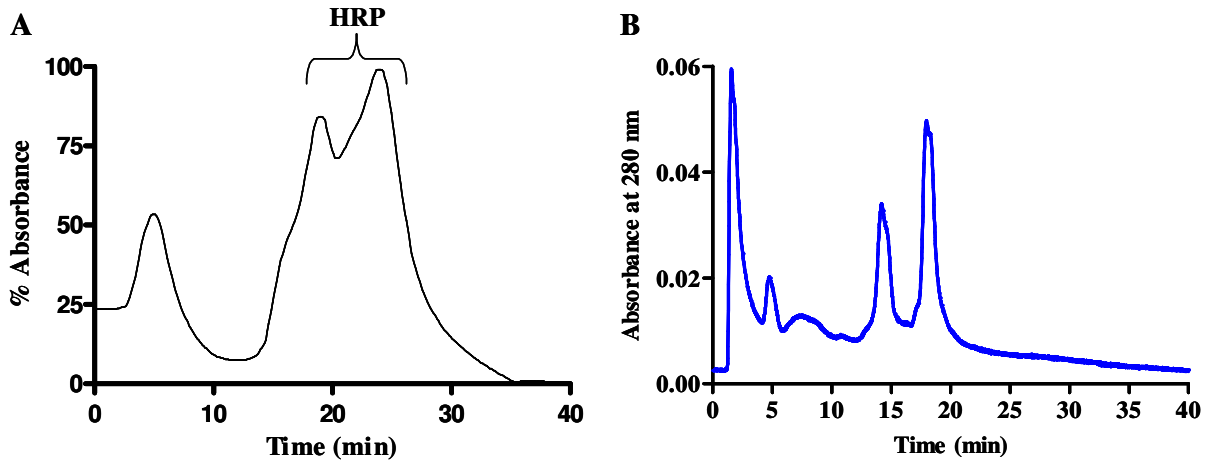

Figure 6.4. ***A***: Elution profile of HRP from the first chromatographic separation in the purification of crude HRP from roots on a CM-Sepharose column. ***B***: Cation-exchange HPLC chromatogram of the fraction containing HRP obtained from the first chromatographic separation. A linear gradient was run from 3% B to 100% B over 25 minutes. *Eluent A*; 0.01 M sodium acetate (pH 4.4). *Eluent B*; 0.01 M sodium acetate, 0.2 M NaCl (pH 4.4). *Sample injected*; 20 µl of HRP-containing fraction.

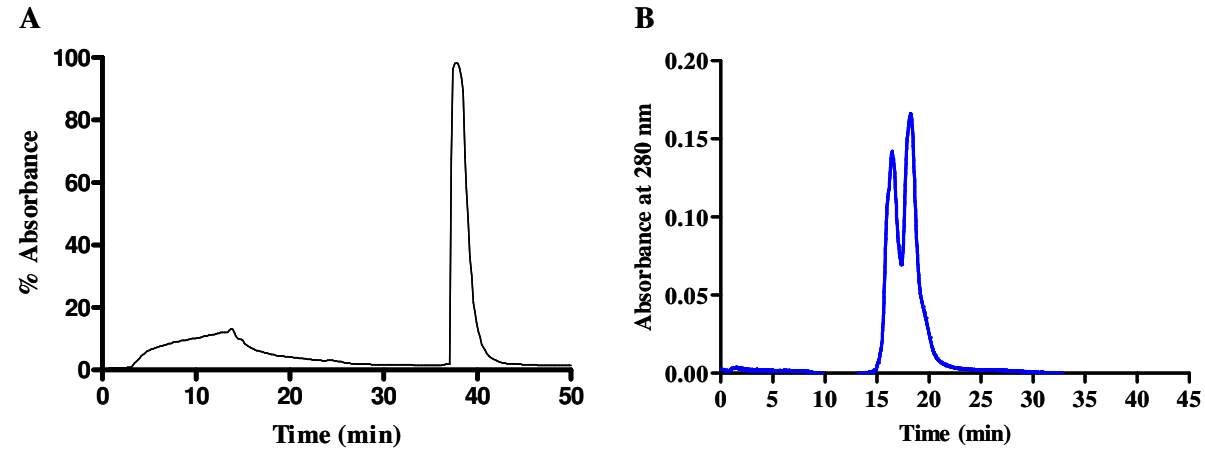

Figure 6.5. ***A***: Elution profile of HRP from the second chromatographic separation in the purification of crude HRP from roots on a CM-Sepharose column. ***B***: Cation-exchange HPLC chromatogram of the fraction containing HRP obtained from the second chromatographic separation. A linear gradient was run from 3% B to 100% B over 25 minutes. *Eluent A*; 0.01 M sodium acetate (pH 4.4). *Eluent B*; 0.01 M sodium acetate, 0.2 M NaCl (pH 4.4). *Sample injected*; 20 µl of 1 HRP-containing fraction.

The abovementioned salt-gradient system had to be adapted for the characterization of apo-HRP given that apo-HRP would not elute using the established cation-exchange HPLC method. Various gradient and isocratic systems were tested and the ionic strength of eluents was varied (results not shown). Optimal retention of apo-HRP was obtained with an isocratic system of 0.01 M acetate, 0.05 M NaCl (pH 4.4) on a CM ion-exchange column (figure 6.6).

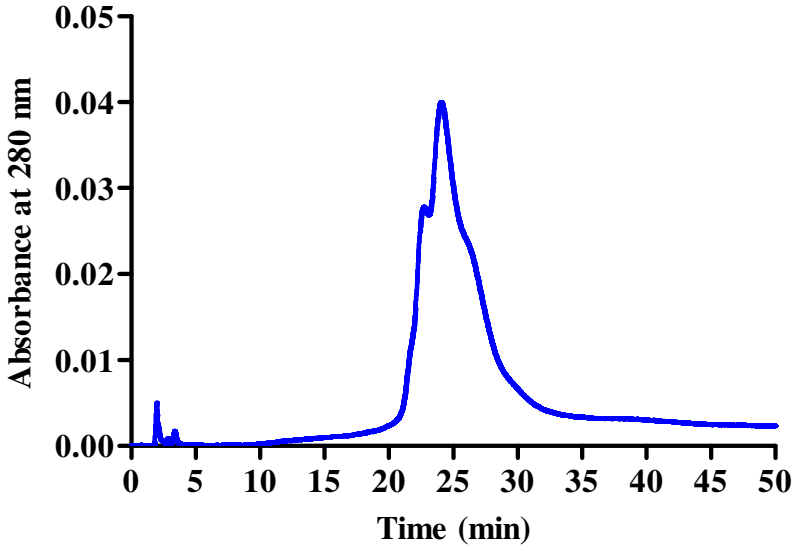

Figure 6.6. Elution profile of apo-HRP from a CM ion-exchange column. An isocratic system was run over 50 minutes. *Eluent A*; 0.01 M sodium acetate, 0.05 M NaCl (pH 4.4). Apo-HRP elutes at 25 minutes. *Sample injected*; 50 µl of 1 mg/ml HRP-C in eluent A.

6.3 Reversed-phase HPLC

Initially, four established separation methods were used to investigate the separation of HRP on a C_{18} column [124]. The methods differed in the composition and initial ratio of the two solvents as well as the gradient systems that were used (see table 10.2). Trifluoric acid (TFA) and acetonitrile were used as solvents. Absorbance was detected at 280 nm and 403 nm, specific for proteins and heme, respectively. A flow rate of 1 ml/min was maintained for all separations. An elution profile of HRP-C for each method is given in figure 6.7 to figure 6.10.

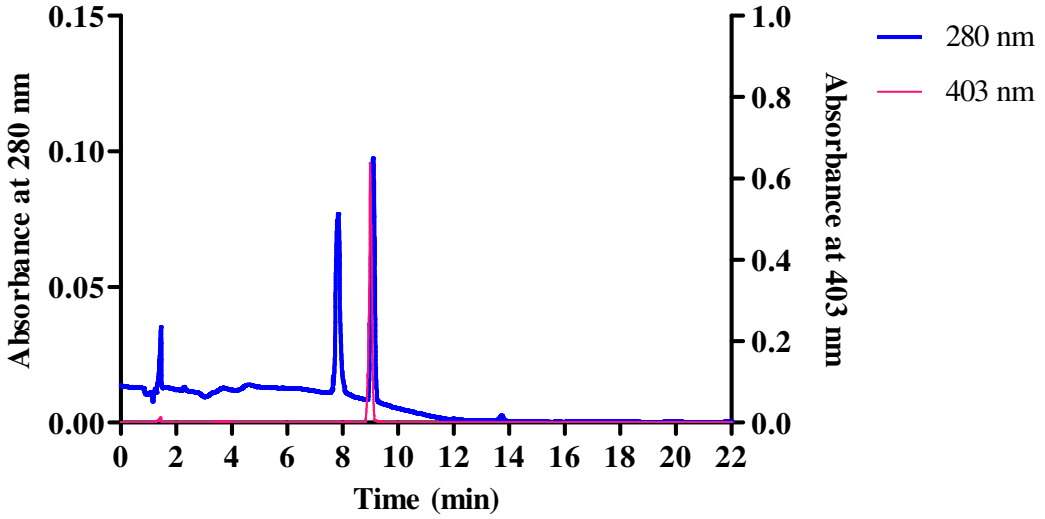

Figure 6.7. Elution profile of HRP-C from an HPLC C_{18} column with separation method 1. A linear gradient was run from 30% B to 100% B over 14 minutes. *Eluent A*; 0.1% TFA. *Eluent B*; 10% eluent A and 90% acetonitrile. *Sample injected*; 15 µl of 1 mg/ml HRP-C in water. HRP-C elutes at 9 minutes.

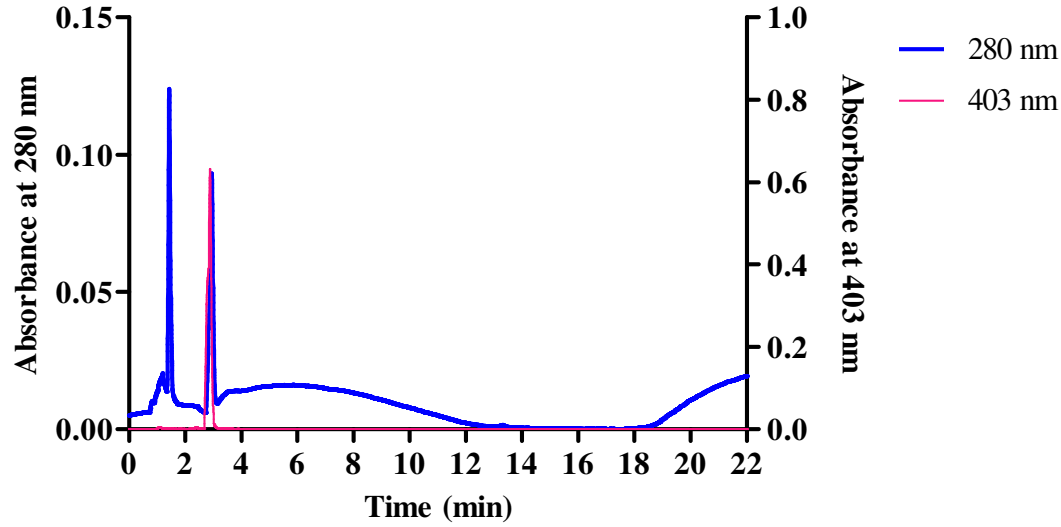

Figure 6.8. Elution profile of HRP-C from an HPLC C_{18} column with separation method 2. A linear gradient was run from 50% B to 100% B over14 minutes. *Eluent A*; 0.1% TFA. *Eluent B*; 10% eluent A and 90% acetonitrile. *Sample injected*; 15 µl of 1 mg/ml HRP-C in water. HRP-C elutes at 3 minutes.

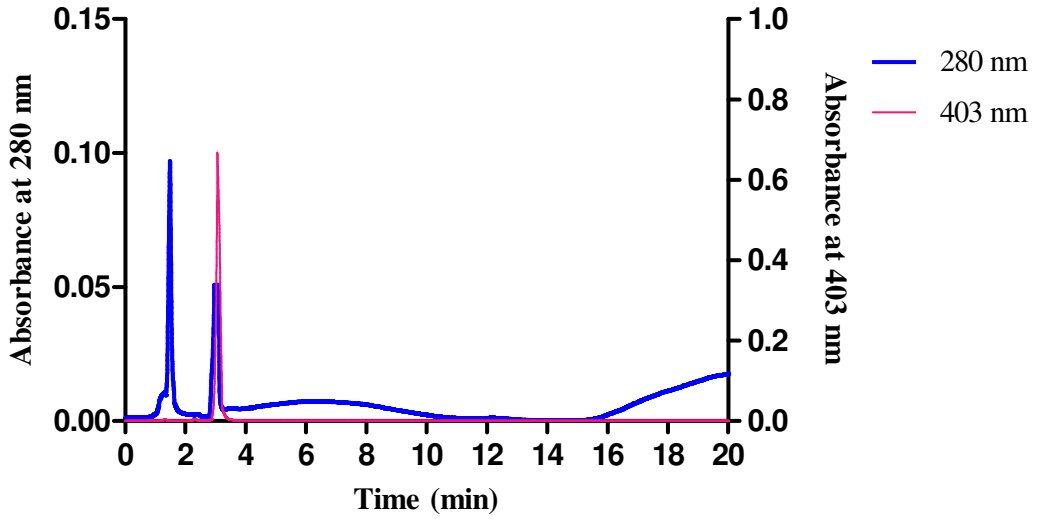

Figure 6.9. Elution profile of HRP-C from an HPLC C_{18} column with separation method 3. A linear gradient was run from 50% B to 100% B over 10 minutes. *Eluent A*; 0.1% TFA. *Eluent B*; 10% eluent A and 90% acetonitrile. *Sample injected*; 15 µl of 1 mg/ml HRP-C in water. HRP-C elutes at 3 minutes.

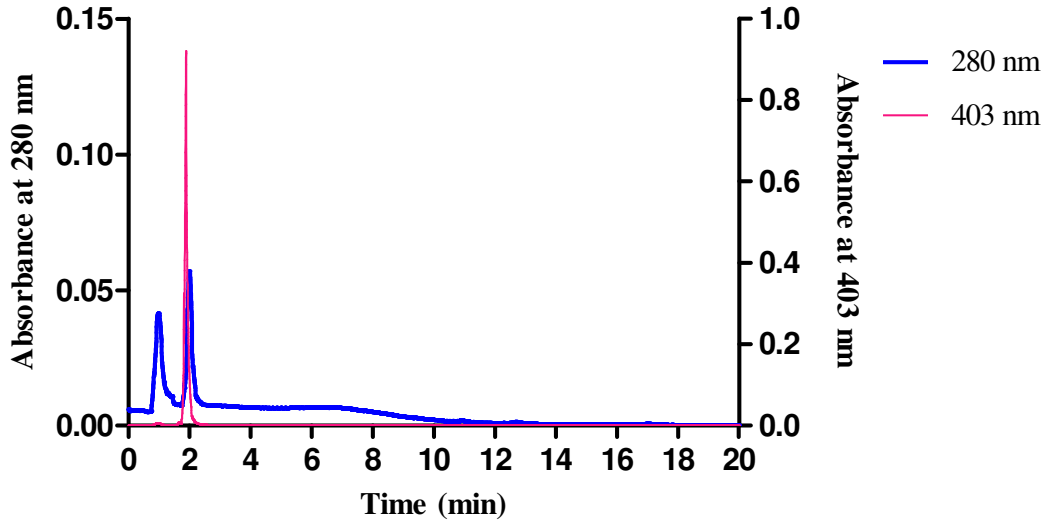

Figure 6.10. Elution profile of HRP-C from an HPLC C_{18} column with separation method 4. A linear gradient was run from 100% A to 100% B over 10 minutes. *Eluent A*; 50% 0.1% TFA and 50% eluent B. *Eluent B*; 10% 0.1% TFA and 90% acetonitrile. *Sample injected*; 15 µl of 1 mg/ml HRP-C in water. HRP-C elutes at 2 minutes.

Separation method 1 produced higher resolution and longer retention of HRP than the other methods and was used to analyse different components and forms of HRP. Apo-HRP, prepared in this study, was analysed using separation method 1 and its elution profile is shown in figure 6.11. The gradient elution time of method 1 was adapted to ensure that the entire sample was eluted before the next sample injection. The elution profiles of an HRP-C standard (provided by Roche), HRP-C purified in this study, crude HRP and heme are shown in figures 6.12 – 6.15. Analyses were done in triplicate and absorbance was measured at 280 nm and 403 nm at a flow rate of 1 ml/min. Reproducible chromatograms could not be obtained for HRP-B (results not shown).

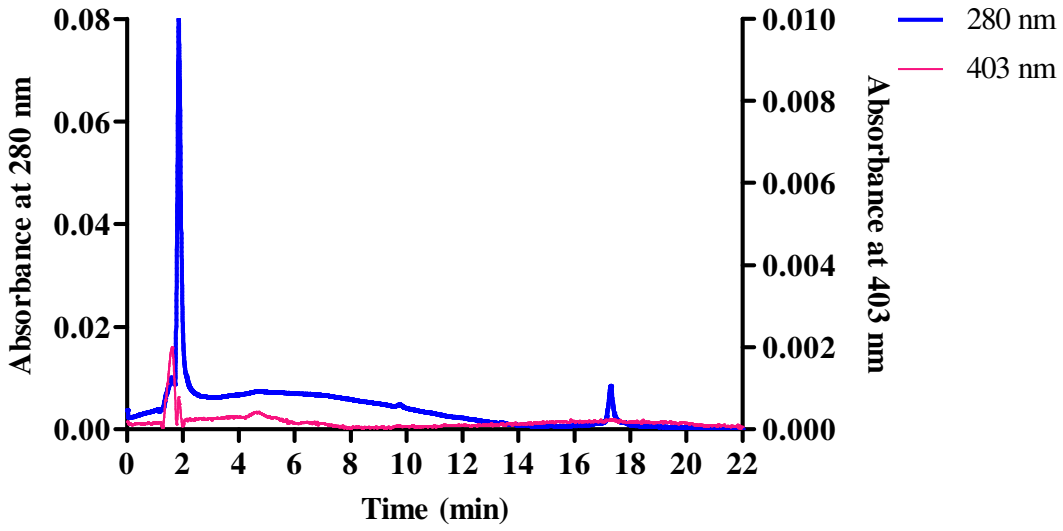

Figure 6.11. Elution profile of apo-HRP from an HPLC C_{18} column with separation method 1. A linear gradient was run from 30% B to 100% B over 14 minutes. *Eluent A*; 0.1% TFA. *Eluent B*; 10% eluent A and 90% acetonitrile. *Sample injected*; 25 µl of 1 mg/ml apo-HRP in water. Apo-HRP elutes at 2 minutes.

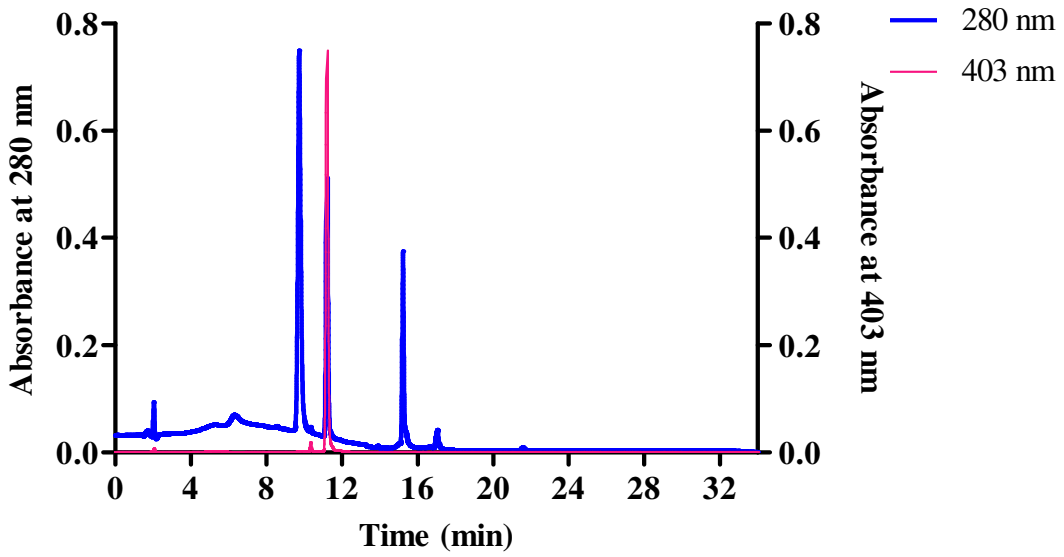

Figure 6.12. Elution profile of the HRP-C standard from an HPLC C_{18} column with adapted separation method 1. A linear gradient was run from 30% B to 100% B over 26 minutes. *Eluent A*; 0.1% TFA. *Eluent B*; 10% eluent A and 90% acetonitrile. *Sample injected*; 25 µl of 1 mg/ml HRP standard in water. HRP elutes at 11 minutes.

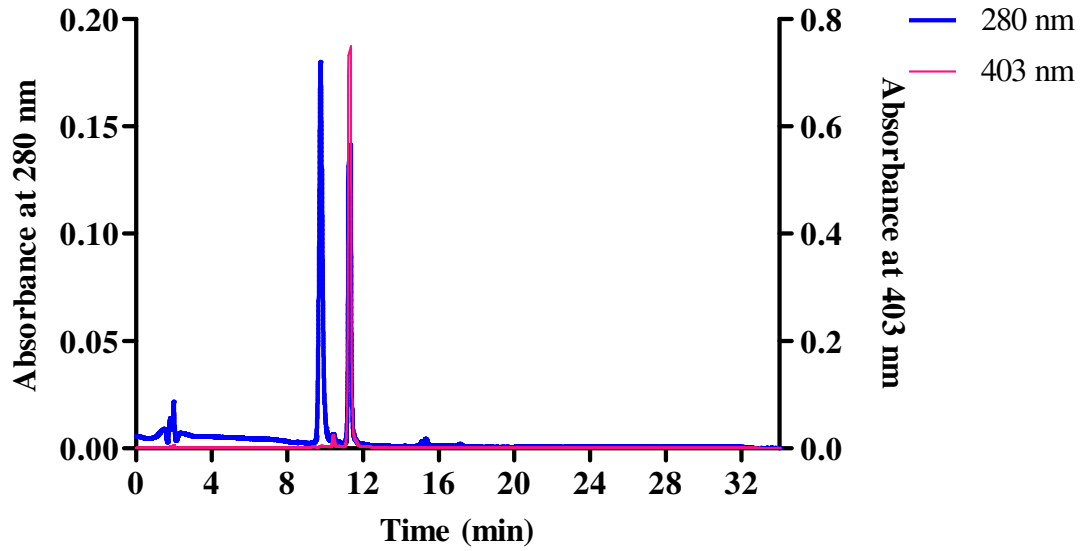

Figure 6.13. Elution profile of purified HRP-C from an HPLC C_{18} column with adapted separation method 1. A linear gradient was run from 30% B to 100% B over 26 minutes. *Eluent A*; 0.1% TFA. *Eluent B*; 10% eluent A and 90% acetonitrile. *Sample injected*; 20 µl of 1 mg/ml HRP-C in water. HRP-C elutes at 11 minutes.

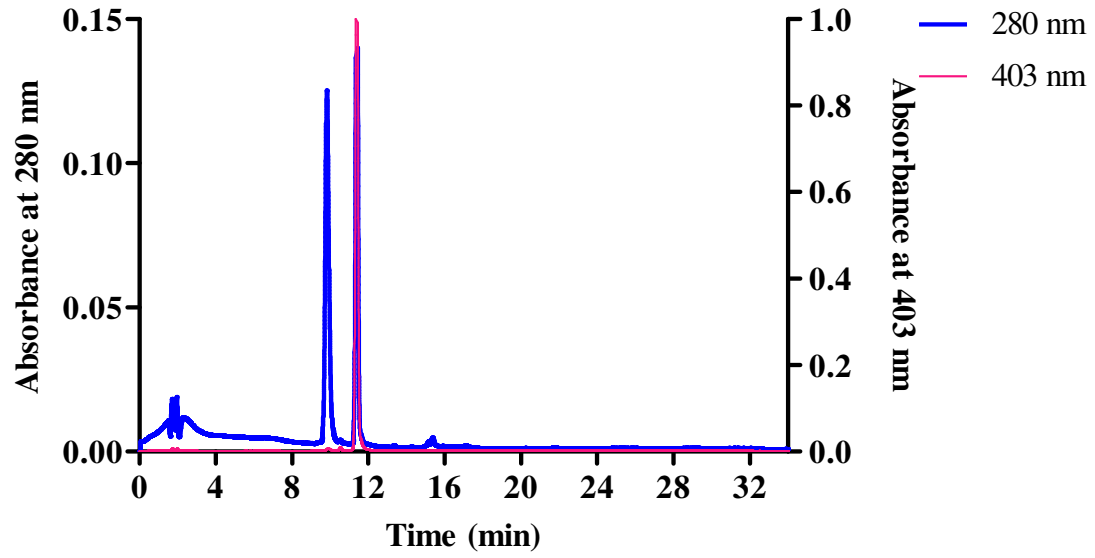

Figure 6.14. Elution profile of crude HRP from an HPLC C_{18} column with adapted separation method 1. A linear gradient was run from 30% B to 100% B over 26 minutes. *Eluent A*; 0.1% TFA. *Eluent B*; 10% eluent A and 90% acetonitrile. *Sample injected*; 20 µl of 1 mg/ml crude HRP in water. HRP-C elutes at 11 minutes.

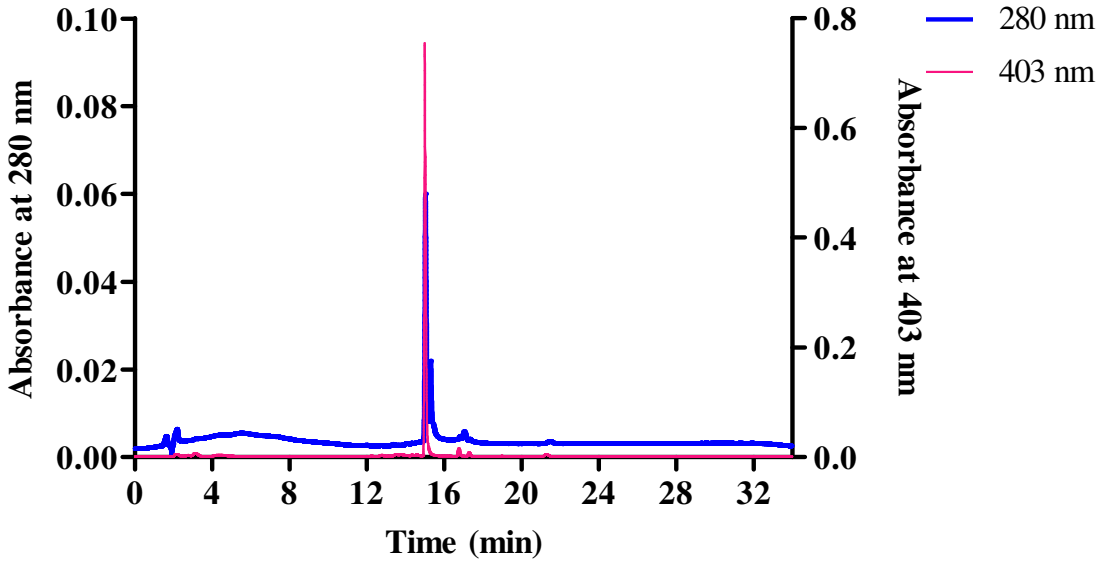

Figure 6.15. Elution profile of heme from an HPLC C_{18} column with adapted separation method 1. A linear gradient was run from 30% B to 100% B over 26 minutes. *Eluent A*; 0.1% TFA. *Eluent B*; 10% eluent A and 90% acetonitrile. *Sample injected:* 10 µl of 0.2 mg/ml heme in ethanol. Heme elutes at 15 minutes.

6.4 Discussion

This study aimed to characterize different components and forms of HRP in terms of ionic and hydrophobic features. Apo-HRP exhibited significantly different chromatographic behaviour to that of the heme-containing form of the enzyme. The established cation-exchange HPLC method did not produce an elution profile for apo-HRP and as a result an isocratic system had to be used. Cation-exchange HPLC analysis of apo-HRP shows that removal of the heme significantly changes the ionic character of the enzyme. From reversed-phase HPLC analysis it is apparent that apo-HRP is less hydrophobic than the active form of HRP. Removal of heme therefore induces major conformational changes in the enzyme.

Analysis of HRP-B by cation-exchange and reversed-phase HPLC did not produce conclusive results. Although protein peaks were detected, reproducible chromatograms could not be obtained. Without a HRP-B standard, positive peak identification is not possible and HRP-B standards are not commercially available at present.

Cation-exchange HPLC analysis of the HPR-C standard and the HRP-C isolated in this study showed a single peak at 16 minutes. The retention time and elution profile of the standard can be used to identify HRP-C on the elution profile of a crude HRP sample (figure 6.3). Integration methods can be used to calculate the area-% and subsequently quantify the relative HRP-C content of a crude sample. Cation-exchange HPLC can also be used to monitor the efficiency of chromatographic steps during purification (figures 6.4 and 6.5). However, this method cannot be used to analyse primary root extracts due to the sensitivity of the ion-exchange column. Primary root extracts contain plant proteins that may have irreversible interactions with the column. Initial salt fractionation and dialysis steps are therefore critical prior to ion-exchange HPLC analysis of samples. While this method is useful for the analysis of samples after certain purification steps, cation-exchange HPLC cannot be used to determine the HRP-C content of primary root extracts.

Reversed-phase HPLC is a more robust analysis method and was investigated as an alternative isoenzyme-detection method due to the constraints of cation-exchange HPLC. A gradient elution system was successfully developed for reversed-phase HPLC analysis of HRP. Once again the HRP-C standard and the HRP-C isolated in this study showed similar elution profiles with a heme-containing protein peak at 11 minutes. Apo-HRP was also characterized and, based on its elution

profile, small amounts of apo-HRP could be detected in the HRP-C standard, the isolated HRP-C and crude HRP.

From this preliminary investigation into the use of HPLC as HRP-isoenzyme detection method, it is evident that the purification and characterisation of other isoenzymes are crucial to the development of such detection methods. This falls beyond the scope of this study and therefore other possible detection methods were explored as described in the following chapter.

7. IMMUNOLOGICAL INVESTIGATION OF HRP

7.1 Introduction

In this study the possibility of using electrophoresis in combination with immunological detection methods as a means to quantify HRP-isoenzyme content in primary root extracts was investigated. The focus of this investigation was the use of either SDS-PAGE or IEF followed by western blot analysis to identify HRP-isoenzymes.

As shown in figure 5.5, no distinction based on molecular mass could be made between HRP-B and HRP-C using SDS-PAGE followed by gel staining. An alternative detection method that was able to differentiate isoenzymes based on properties other than molecular mass was therefore needed. One such method is detection by immunoblotting. For immunological identification of isoenzymes in a primary root extract after electrophoresis antibodies specific for respective isoenzymes need to be produced. It was postulated that, if these antibodies can be produced, isoenzyme content of primary root extracts could be quantified after electrophoresis by means of western blot analysis.

For these immunological investigations antibodies were raised against both the HRP-B and the HRP-C purified in this study (see chapter 5.4). These antibodies were used in western blots to determine, firstly, if the antibodies could detect the isoenzyme it was raised against and secondly, whether the antibodies could discriminate between the two isoenzymes. The production of antibodies against HRP was carried out as previously described by Bellstedt *et al.* [125]. A schematic presentation of this immunization method is given in figure 7.1.

In short, a mild acid treatment is used to prepare naked bacteria (NB) from *Salmonella minnesota R595* to use as immune carriers. The acid treatment creates a hydrophobic outer surface on the bacteria to which antigens, such as proteins, can be readily adsorbed. The resulting complexes are subsequently used to raise antibodies against the adsorbed proteins which now act as antigenic determinants.

Prior to western blot analysis, the antigen (HRP) was electrophoresed by either SDS-PAGE or IEF. While standard electrophoretic techniques, such as SDS-PAGE, are used for separations at a constant pH, IEF may be defined as electrophoresis in a pH gradient. IEF employs a pH gradient to separate amphoteric molecules according to differences in pI. The pH gradient is established

between two electrodes and is stabilized by carrier ampholytes. During IEF a protein migrates in the pH gradient until it aligns at its pI-value, at which point the protein possesses no net overall charge. The protein concentrates at its pI-value as migration ceases. Due to the reported difference in pI for HRP-isoenzymes, this study proposed that isoenzymes in primary root extracts could be resolved with IEF and quantified by western blot analysis.

After electrophoresis, whether by SDS-PAGE or IEF, the antigen (HRP) was subsequently electro-transferred from the gel to a nitrocellulose membrane. This membrane was incubated with primary antibody (anti-HRP) which recognizes the antigen and binds to it. The primary antibody was subsequently recognized by an antibody-enzyme complex. The enzyme from the antibody-enzyme complex reacts with a specific substrate and converts it to a chemiluminescent product which forms the recognition system of the blot. Immunoblotting is a sensitive, specific method that can be used to determine small amounts of an isolated protein as well as a specific protein in a crude preparation.

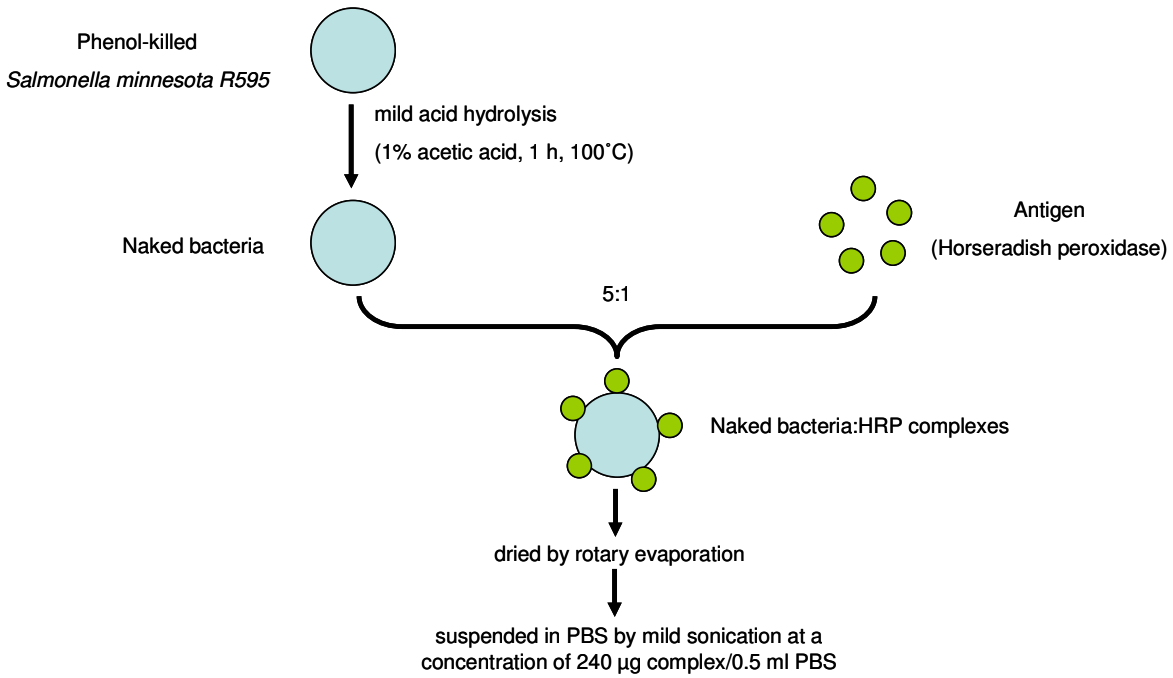

Figure 7.1. Schematic representation of use of naked bacteria to produce antibodies against horseradish peroxidase isoenzymes [adapted from 125]

7.2 Production of antibodies against HRP

HRP-B and HRP-C were prepared using cation-exchange chromatography as described in chapter 5.3. Purified HRP-B and HRP-C were subsequently adsorbed to NB, using the following procedure. A suspension of NB in distilled water was prepared by homogenization with a loosely fitting Teflon homogenizer. The NB suspension and the respective isoenzyme were subsequently mixed in a 5:1 ratio (dry mass). The resulting suspension was dried on a rotary evaporator and the resulting complexes re-suspended by brief sonication in phosphate buffered saline (PBS) to give a concentration of 240 µg/0.5 ml. The NB:isoenzyme complex was used to induce an immune response in rabbits.

Rabbits were immunised with either HRP-B or HRP-C complexes. Immunisation was carried out over a 49-day period on days 0, 3, 8, 14, 17, 21, 42, 45 and 49. Rabbits were immunised with 0.5 ml of a 240 µg NB:isoenzyme complex/0.5 ml suspension i.e. 40 µg HRP-isoenzyme adsorbed to 200 µg NB dissolved in PBS buffer (pH 7.2). Venous blood was collected prior to immunisation on day 0 (2 ml) and on days 28 (2 ml) and 56 (24 ml). The blood was allowed to clot. The antiserum was prepared by centrifugation at 4ºC. Antiserum was stored at -20ºC.

Isoenzyme recognition by the antibodies was investigated by ELISA, performed as previously described [126]. Briefly, microtitre plates were coated with either HRP-B or HRP-C (5 µg isoenzyme per well). The HRP-coated wells were incubated with equal volumes 0.1 M NaN_3 and 0.1 M H_20_2 for 90 minutes at room temperature to inactivate the bound isoenzymes [69]. After incubation, the wells were washed several times with PBS-Tween solution and subsequently the ligand-free surface of the wells was saturated by incubation with casein buffer at 37˚C. Several wash steps were included after saturation, followed by incubation with either an anti-HRP-B or anti-HRP-C dilution series in casein buffer containing Tween-20. The wells were washed several times and 100 µl goat anti-rabbit HRP-conjugated secondary antibody (10 000x dilution in casein buffer containing Tween-20) was added per well. Unbound secondary antibody was removed by wash steps and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate solution was added to the wells. Absorbance was detected at 405 nm. The titre value for each day 56 antiserum was determined as the serum dilution which produces an absorbance 0.1 (figure 7.2 and 7.3). The titre determined for anti-HRP-B recognition of HRP-B was 2 000 and for anti-HRP-C recognition of HRP-C was 11 000 (titre values rounded up to the nearest 100).

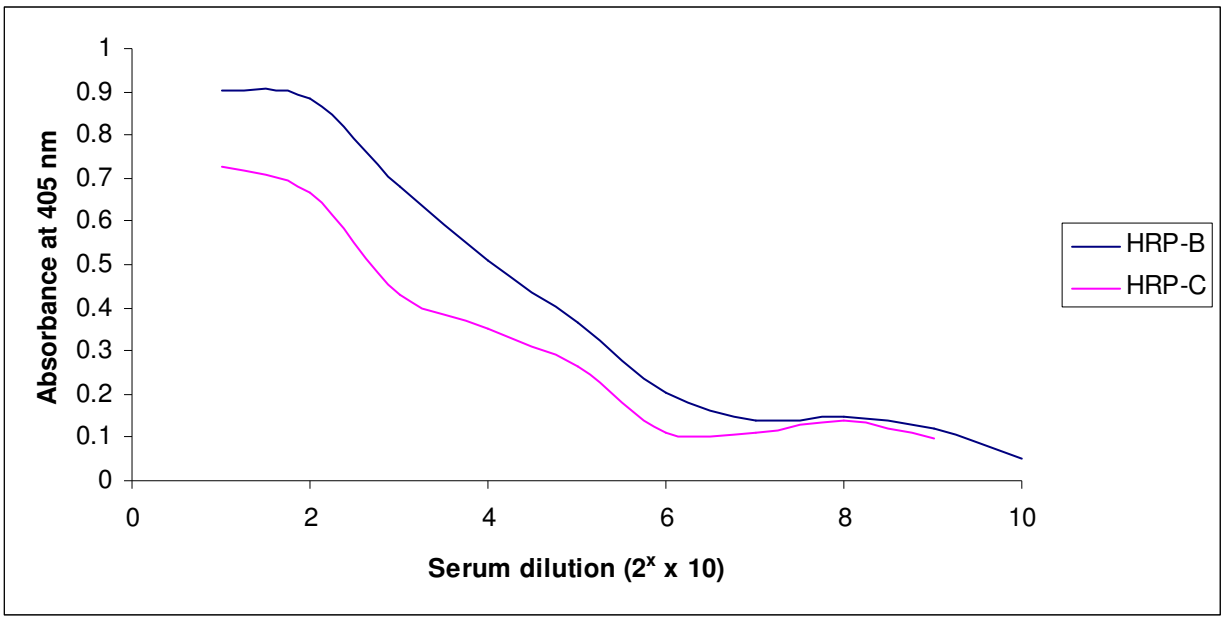

Figure 7.2. Absorbance of anti-HRP-B dilutions against HRP-B and HRP-C. Titre value determined for anti-HRP-B was approximately 2 000.

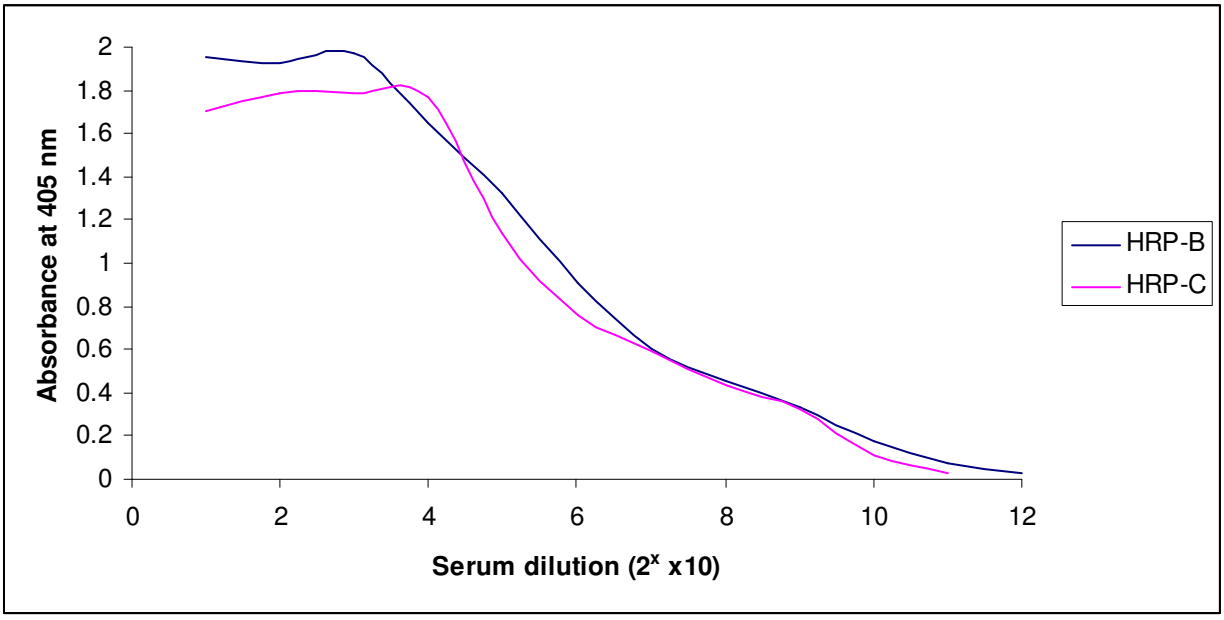

Figure 7.3. Absorbance of anti-HRP-C dilutions against HRP-B and HRP-C. Titre value determined for anti-HRP-C was approximately 11 000.

7.3 Western blot analyses

Purified HRP-B and HRP-C were subjected to either SDS-PAGE or IEF. The isoenzymes were then transferred from the respective gels to nitrocellulose membranes using an electro-transfer procedure. Western blot procedures were performed on the membrane as previously described [127]. The electrotransfer of isoenzymes from gels to nitrocellulose membranes was monitored by colouring the nitrocellulose membranes with Ponceau-S stain for approximately two minutes. Transferred isoenzymes could be detected as temporary red bands which could be washed away with PBS-Tween.

7.3.1 SDS-PAGE and western blot

The electrophoretic analysis of clarified extract, purified HRP-B and HRP-C and a HRP-C standard (provided by Roche) on a 15% poly-acrylamide gel is shown in figure 7.4. Clarified extract refers to the filtrate produced by the homogenization of roots and subsequent filtration of this homogenate (see chapter 5.2). The clarified extract used in these experiments was prepared from one kg of frozen roots in three volumes of 0.4 M NaCl. Staining with Coomassie Brilliant Blue revealed a single band for the HRP-isoenzymes as well as the HRP-C standard. Two bands could be seen for the clarified extract. A molecular mass marker was also loaded on the gel.

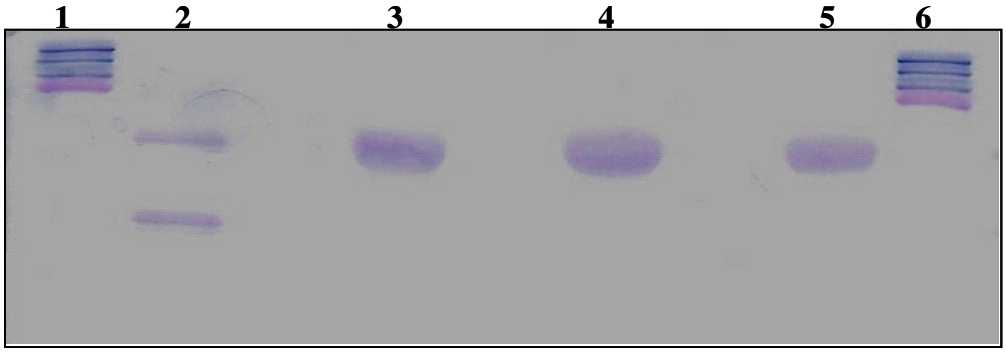

Figure 7.4. SDS-PAGE of HRP-isoenzymes and HRP in clarified extract. Isoenzymes were resolved under denaturing conditions on 15% poly-acrylamide gel. *Lane 1;* Mr marker (10-250 kDa). *Lane 2;* 20 µl clarified extract (approximately 40 mg protein). *Lane 3;* 20 µg HRP-B. *Lane 4;* 20 µg HRP-C. *Lane 5;* 20 µg HRP-C standard. *Lane 6;* Mr marker (10-250 kDa).

The abovementioned electrophoresis was repeated without staining and the isoenzymes were transferred from the gels to nitrocellulose membranes using an electro-transfer procedure (see chapter 10.13.1). After electro-transfer, the nitrocellulose membrane was incubated overnight with casein buffer to prevent non-specific binding. The membrane was subsequently incubated at 37ºC for one hour with appropriate dilutions of the rabbit-anti-HRP-B or rabbit-anti-HRP-C antiserum, respectively. The membrane was washed four times with PBS-Tween after this step and after all subsequent incubation steps. After incubation with the primary antibody and subsequent washing steps, the membrane was incubated at 37ºC for one hour with a solution of goat anti-rabbit immunoglobulin (IgG)-HRP conjugate in casein buffer. After final washing steps the membrane was incubated with enhanced chemiluminescence substrate for 1 minute before exposure by hypercasette.

The western blot analysis of HRP-B, HRP-C, HRP-C standard and clarified extract with anti-HRP-B and anti-HRP-C, respectively is shown in figure 7.5. A 5 000x dilution of anti-HRP-B in casein buffer and 20 000x dilution of anti-HRP-C in casein buffer were used for these respective blots. For both blots a 5 000x dilution of goat-anti-rabbit HRP-conjugated antibody in casein buffer was used. Exposure of the X-ray film by hypercasette was for one minute.

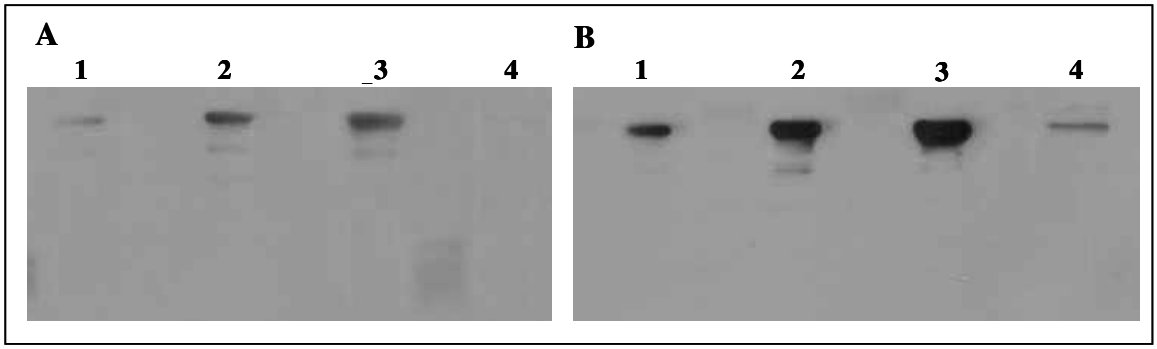

Figure 7.5. Western blot analysis of isolated HRP-isoenzymes and HRP in clarified extract after SDS-PAGE. Isoenzymes were separated under denaturing conditions on 15% poly-acrylamide gel before electrotransfer to nitrocellulose membranes. *Lane 1;* 5 µg HRP-B. *Lane 2;* 5 µg HRP-C. *Lane 3;* 5 µg HRP-C standard. *Lane 4;* 20 µg protein in clarified extract. ***A***. Western blot analysis with anti-HRP-B. Proteins were detected using a 5 000x dilution of polyclonal rabbit anti-HRP-B sera. ***B***. Western blot analysis with anti-HRP-C. Proteins were detected using a 20 000x dilution of polyclonal rabbit anti-HRP-C sera.

7.3.2 Isoelectric focusing and western blot

HRP was separated on 5% Servalyt™ PreNets™ using a horizontal electrofocusing system. Electrofocusing experiments were carried out at constant power (4 W). The current was set at a maximum value of 250 mA and voltage was set at a maximum value of 1200 V. Electrofocusing under these conditions would last between 2 and 4 hours. After electro-focusing, Servalyt™ PreNets™ were stained or the resolved proteins were transferred using an electro-transfer system.

A Servalyt™ PreNet stained with Coomassie Blue R250 after electrofocusing is shown in figure 7.6. For staining purposes the gel remained attached to the Gel-Fix™ support film. The gel was submerged in fixing solution before being submerged in Coomassie Blue R250 staining solution. The gel was destained for an hour in a solution containing 10% acetic acid and 30% ethanol. Different quantities of the HRP-C standard were loaded on the gel shown in figure 8.6. A marker, containing a mixture of eight proteins with well-defined pI-values ranging between 3.6 and 9.3, was also loaded. A standard curve was constructed and approximate pI-values of the HRP-C standard were determined (see chapter 10.10.6). The pI-values of the two bands seen after electrofocusing of the HRP-C standard were approximately 7.1 and 7.7, respectively.

These pI-values were lower than most reported pI-values for HRP-C. Although pI-values ranging between 8.8 and 9.6 are widely reported for HRP-C, some studies have reported pI-values as low as 7.7 [56]. To test the validity of the IEF system used in this study, another gel was used to resolve crude HRP and the HRP-C isolated in this study (figure 7.7). Chymotrypsin was also loaded on the gel as a standard; the reported pI-values for chymotrypsin range between 8.1 and 9.1. A standard curve was again constructed to determine the approximate pI-values of the proteins resolved in this electrofocusing experiment (see chapter 10.10.7). The pI-values corresponding to the two visible bands from the crude HRP and HRP-C isolated in this study were 7.2 and 7.8, respectively. Approximate pI-values corresponding to the two visible bands of chymotrypsin were 8.9 and 9.2.

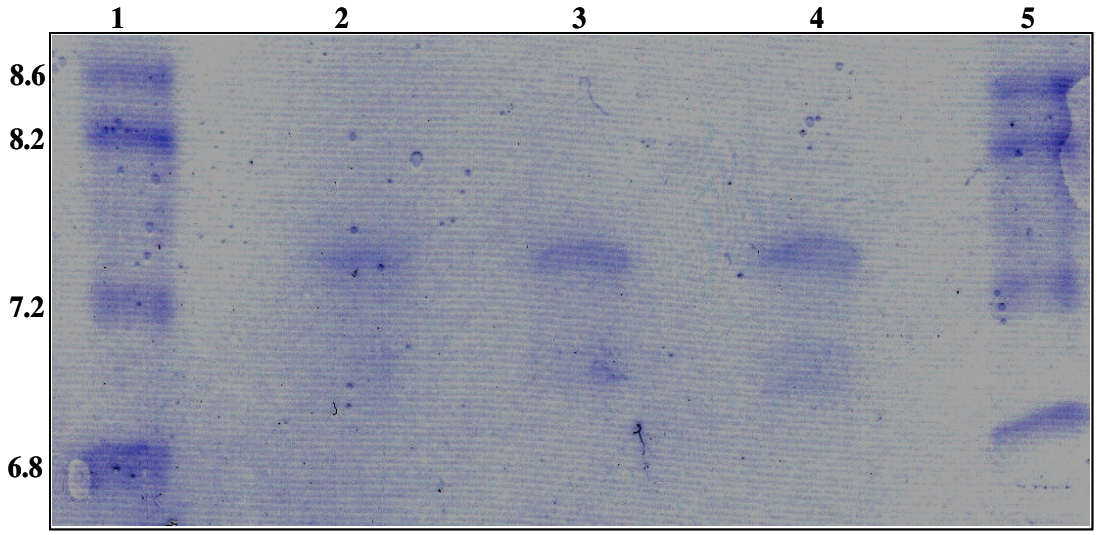

Figure 7.6. Isoelectric focusing of HRP-C standard. *Lane 1*; marker with pH assigned to the left. *Lane 2*; 5 µg HRP-C standard. *Lane 3*; 10 µg HRP-C standard. *Lane 4*; 15 µg HRP-C standard. *Lane 5*; marker. pI–values corresponding to the visible bands in the HRP-C standard were 7.1 and 7.7.

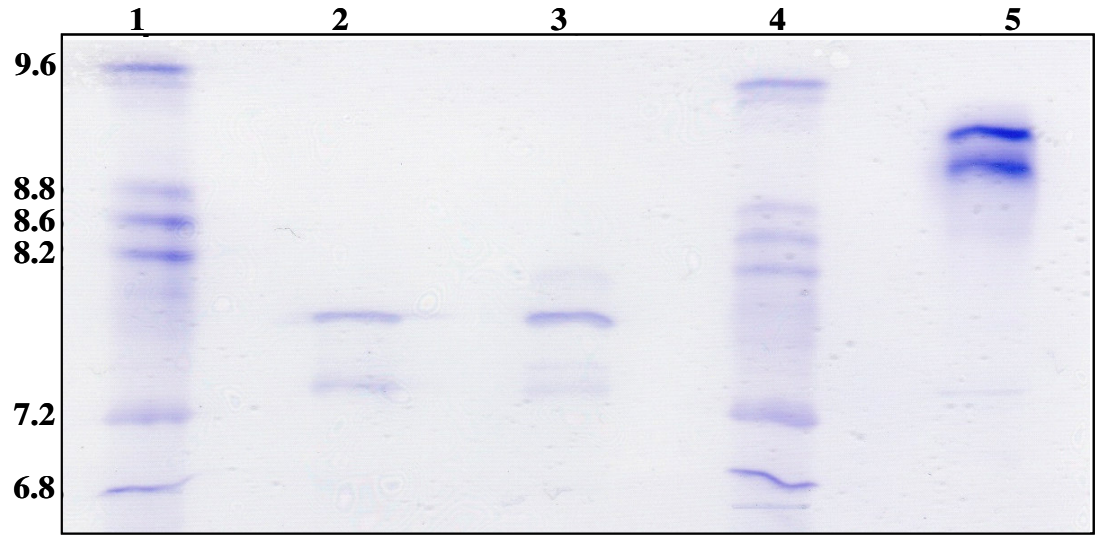

Figure 7.7. Isoelectric focusing of crude HRP and HRP-C. *Lane 1*; marker with pH assigned to the left. *Lane 2*; 5 µg crude HRP. *Lane 3*; 5 µg HRP-C. *Lane 4*; marker. *Lane 5*; 5 µg chymotrypsin. pI-values corresponding to the visible bands in crude HRP and HRP-C was 7.2 and 7.8. pI-values corresponding to the visible bands in chymotrypsin were 8.9 and 9.2.

Subsequently, IEF was used in combination with electro-transfer and western blot analysis to identify HRP-isoenzymes. After electrofocusing, isoenzymes were transferred to a nitrocellulose membrane using a semi-dry transfer system. Prior to electro-transfer, the nitrocellulose membrane was soaked in semi-dry transfer buffer. The gel was placed on the nitrocellulose membrane and the Gel-Fix™ support film was removed. The gel and membrane were sandwiched between layers of thick blotting paper soaked in semi-dry transfer buffer. Electro-transfer was carried out at 4 V for 60 minutes. After electro-transfer non-specific binding to the membrane was blocked overnight at 4ºC using casein buffer.

The western blot analyses of HRP-B, HRP-C and crude HRP after electrofocusing under conditions as mentioned above is shown in figure 7.8. For both blots a 20 000x dilution of anti-HRP-C in casein buffer, followed by 5 000x dilution of goat anti-rabbit IgG-HRP conjugate in casein buffer, was used. Exposure of the X-ray film by hypercasette was for one minute.

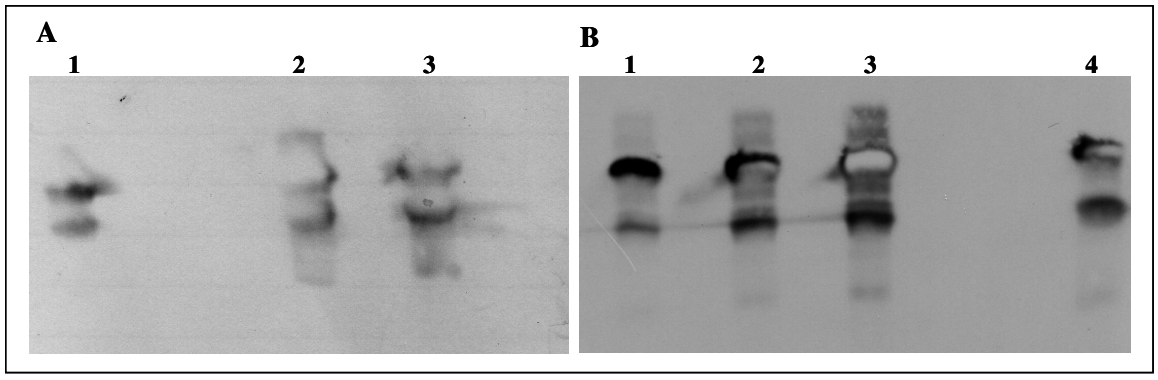

Figure 7.8. Western blot analyses of isolated HRP-isoenzymes and crude HRP after isoelectric focusing. Proteins were resolved on 5% Servalyt™ PreNets™ before electrotransfer to nitrocellulose membranes. *Primary antibody*; 20 000x dilution of polyclonal rabbit anti-HRP-C. ***A***. *Lane 1;* 5 µg HRP-B. *Lane 2;* 5 µg crude HRP. *Lane 3;* 5 µg HRP-C. ***B.*** *Lane 1;* 2.55 µg crude HRP. *Lane 2;* 5 µg crude HRP. *Lane 3;* 10 µg crude HRP. *Lane 5*; 5 µg HRP-C.

7.4 Discussion

In this study the use of two electrophoretic methods in combination with immunological detection methods as possible means to determine HRP-isoenzyme content in primary root extracts was investigated.

Antibodies specific for HRP-B and HRP-C, respectively were raised in rabbits. These antibodies were used in western blot analysis. The use of SDS-PAGE followed by western blot analysis was investigated first. HRP-isoenzymes were separated according to molecular mass using SDS-PAGE. It was postulated that even though no differences in molecular mass between HRP-B and HRP-C could be detected with SDS-PAGE, antibodies specific for the respective isoenzymes would allow the detection and quantification of these isoenzymes in raw material.

From figures 7.2 – 7.4 it is evident that the antibodies produced in this study show a degree of cross-reactivity i.e. the anti-HRP-B and anti-HRP-C were able to detect both HRP-B and HRP-C. When the dilution factors of each antiserum used in western blot analysis are considered, anti-HRP-C recognizes both HRP-C and HRP-B more readily than anti-HRP-B. There are many possible explanations for the apparent cross-reactivity of anti-HRP sera. Despite the high degree of purity shown for each of the isolated isoenzymes used in immunisation, contamination cannot be ruled out completely. More than 30 multiple forms of HRP-isoenzymes have been reported; it is therefore possible that the isolated isoenzymes might contain different forms of the specific isoenzyme. Additionally, little is known about the difference in glycan content and glycosylation sites between isoenzymes. It is probable that HRP-isoenzymes share a number of antigen determinates. It is therefore concluded that, unless antibodies specific for respective isoenzymes can be produced, SDS-PAGE combined with western blot analysis cannot be used to quantify isoenzyme content of a primary root extract.

Subsequently, it was postulated that if HRP-isoenzymes in a primary root extract could be separated by pI differences, western blot analysis using antibodies that detect HRP could be used to quantify isoenzyme content. Previous studies have shown the successful separation of HRP-isoenzymes according to respective pI-values using IEF [2,128]. Separation of isolated isoenzymes and crude HRP by IEF in this study showed lower pI-values for HRP than reported in the literature. Although pI of HRP-isoenzymes have been shown to differ, the experimental conditions when determining pI play a major role in the resulting values. Most studies that report pI for HRP-isoenzymes

determined these values under experimental conditions that differed significantly from those used in this study. These studies generally used thicker, self-cast gels with narrow pH gradients. This study was limited to the use of precast IEF gels with wide pH gradients for blotting purposes.

In figure 7.8 two definite bands can be seen for HRP-B, HRP-C and crude HRP. Further characterization of HRP-isoenzymes is needed to identify the specific isoenzyme present in these bands. Also, an IEF profile for all isoenzymes should be established with a standardized protocol. With further investigation, IEF combined with western blot analysis could be developed into an isoenzyme quantification technique. IEF is, however, an expensive, labour-intensive technique and is not, therefore, a practical solution to isoenzyme quantification in industrial production of HRP.

Even though manufacturers are still unable to determine the isoenzyme content and subsequent commercial value of roots, the large scale production of HRP continues due to the demand for HRP in clinical, industrial and diagnostic applications. The last part of this study focused on the development of one such application of HRP: a melamine quantification immunoassay.

8. DEVELOPMENT OF A MELAMINE-DETECTION IMMUNOASSAY

8.1 Introduction

As mentioned in chapter 3, HRP is commonly used in immunoassays for quantitative assessment of the concentration a specific analyte. A very topical example of such an assay is a solid-phase immunobinding assay for the assessment of melamine content in food samples. In 2007, a large scale recall of certain pet foods was issued internationally due to melamine contamination and subsequent animal deaths. This was followed by the Chinese milk scandal in 2008 where several companies were implicated in the adulteration of powered milk formula which led to the hospitalization and death of several infants. Due to these recent health scares the development of fast, cost-effective melamine quantification methods has become crucial.

Current methods for melamine quantification include HPLC, solid-phase extraction followed by LCMS and electrospray ionization methods coupled to mass spectroscopy [129,130]. Although these methods allow accurate and sophisticated analysis of samples, the technology is not always readily available to food manufacturers. Quantification by immunoassays is more accessible and can be a valuable tool in routine analysis. Immunoassays for melamine-detection are commercially available but are expensive. The final aim of this study forms part of the development of a more affordable melamine-detection immunoassay using HRP.

As discussed, HRP is an ideal candidate for use in immunoassays due to its robustness and the relatively low costs of its production. The proposed immunoassay is a derivative of two-site ELISA techniques (figure 8.1). In brief, melamine-specific antibodies are used to coat microtitre plates. Unbound antibodies are removed by a wash step and excess binding sites are blocked with an irrelevant protein. After this blocking step, the plate is incubated with a sample of unknown melamine concentration together with a solution of melamine coupled to HRP. Excess sample and melamine-HRP is removed and a colorimetric substrate of the enzyme is added. The melamine concentration of the sample can therefore be determined spectrophotometrically; the higher the concentration of melamine in the sample, the less melamine-HRP is present in the well and therefore lower absorbance will be detected. The development of this immunoassay is therefore dependant on the production of melamine-specific antibodies and the successful coupling of melamine to HRP.

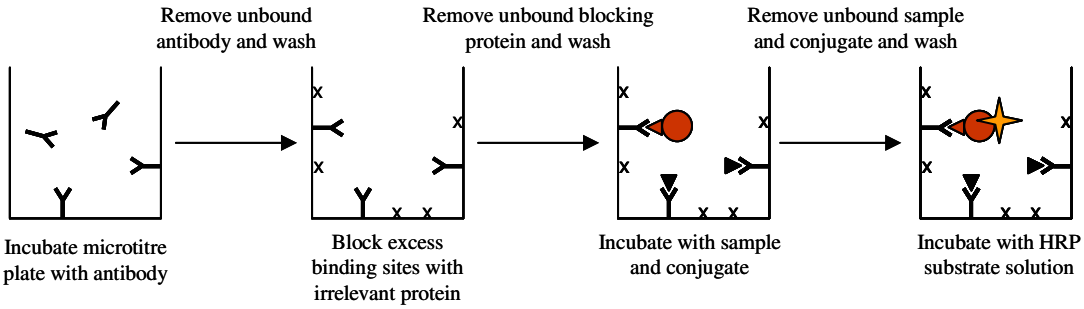

Figure 8.1. Schematic representation of melamine-detection immunoassay using HRP.

Two possible methods for the coupling of melamine to HRP were investigated. Firstly, it was hypothesized that melamine could be coupled to HRP through the formation of amide bonds between the primary amine groups of melamine and the carboxylic acids of HRP. As mentioned in chapter 2, HRP-C contains considerable amounts of aspartic acid and glutamic acid residues (see table 2.3). Amide bond formation by direct reaction of amines with carboxylic acids is often unsuccessful and therefore the carboxylic acid is usually treated with a carbodiimide to improve its ability to accept protons. A variety of commercial carbodiimides are available for use as carboxyl activating agents and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was selected for the purpose of this study. EDC, a water-soluble carbodiimide, is commonly used in organic chemistry to activate carboxylic acids towards the coupling of primary amines to yield amine bonds in the pH 4.0 – 6.0 range.

In brief, the formation of an amine bond by reaction of an amine and a carboxylic acid in the presence of EDC can be described as follows. Firstly, EDC is protonated by the carboxylic acid and the resulting carboxylate then adds to the C=N bond of the protonated carbodiimide, yielding a reactive acylating agent. Nucleophilic acyl substitution by the amine ensues and results in amide bond formation between the amine and carboxylic acid. In theory, this would then couple the amine groups of melamine to HRP.

The second coupling method proposed in this study involves the reaction of a primary amine with aldehydes produced by the oxidation of the carbohydrates on the enzyme surface. An oxidizing agent, in this case sodium periodate, is used to oxidize primary alcohols on the carbohydrate chains to aldehydes. Subsequently the primary amines of melamine undergo nucleophilic addition to aldehydes and result in substituted imines.

8.2 Coupling by reaction of an amine and carboxylic acid with EDC

Reactions using different HRP:EDC:melamine ratios were evaluated. HRP-C purified in this study was used to prepare a 20 mg/ml enzyme solution in 25 mM sodium phosphate buffer (pH 5.0). The concentration of EDC solutions and melamine solutions used in the reactions were varied. EDC solutions were prepared in 25 mM sodium phosphate buffer (pH 5.0) while melamine was dissolved in 0.4 M sodium phosphate buffer (pH 8.0). Equal volumes of HRP and EDC solutions were added together and incubated at room temperature for 5 minutes. The HRP:EDC solution was then slowly added to an equal volume of melamine solution. The final solution was incubated overnight on a shaker at room temperature. After incubation, the final solution was transferred to a G10-Sephadex column (1 x 5 cm) and allowed to elute under gravity. The resulting solution was freeze-dried and subsequently analyzed by LCMS. A sample of the HRP-C used in this experiment was also analyzed by LCMS as a control (figures 8.2 – 8.4).

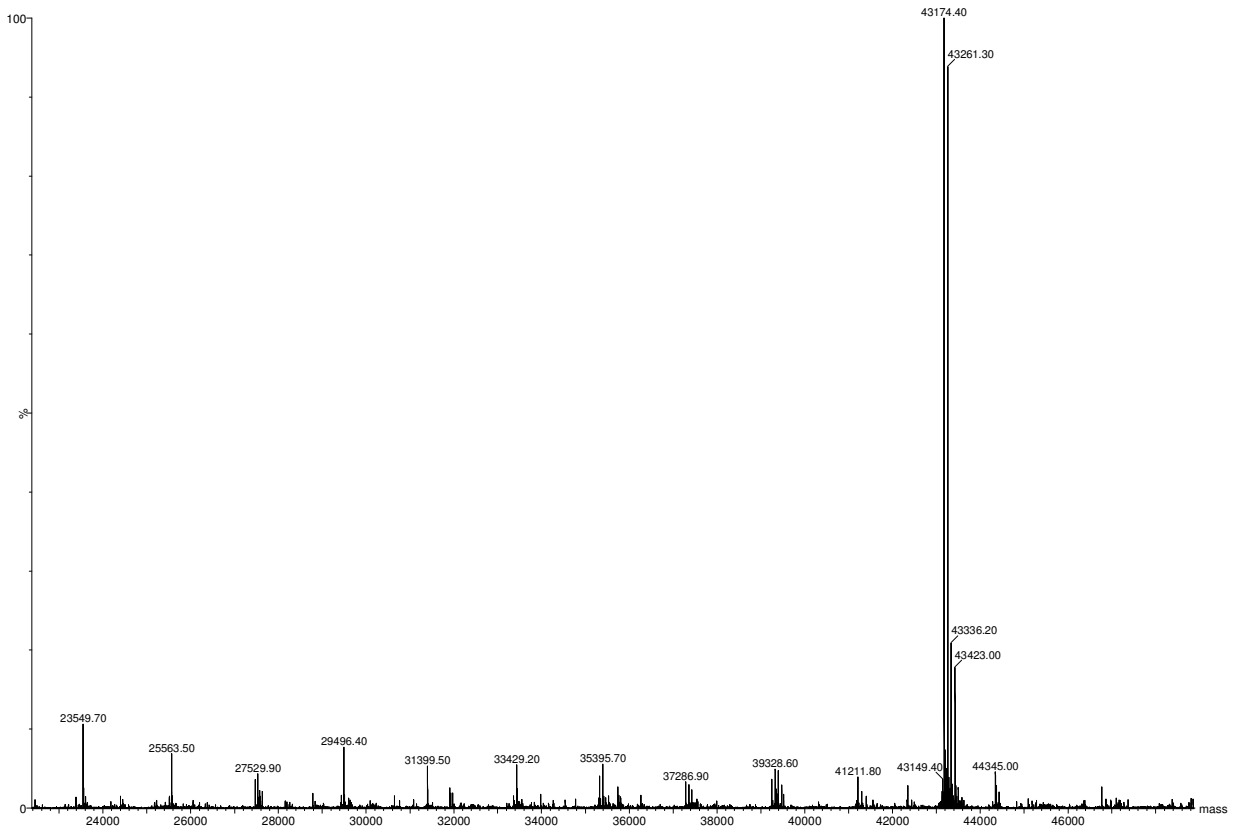

Figure 8.2. Full mass spectrum of HRP-C by LCMS analysis. Separation was carried out on a C_{18} column with an acetonitrile gradient containing 0.1 % formic acid and detected by ESI-mass spectrometer in positive mode. The molecular mass of HRP is 43 174.40.

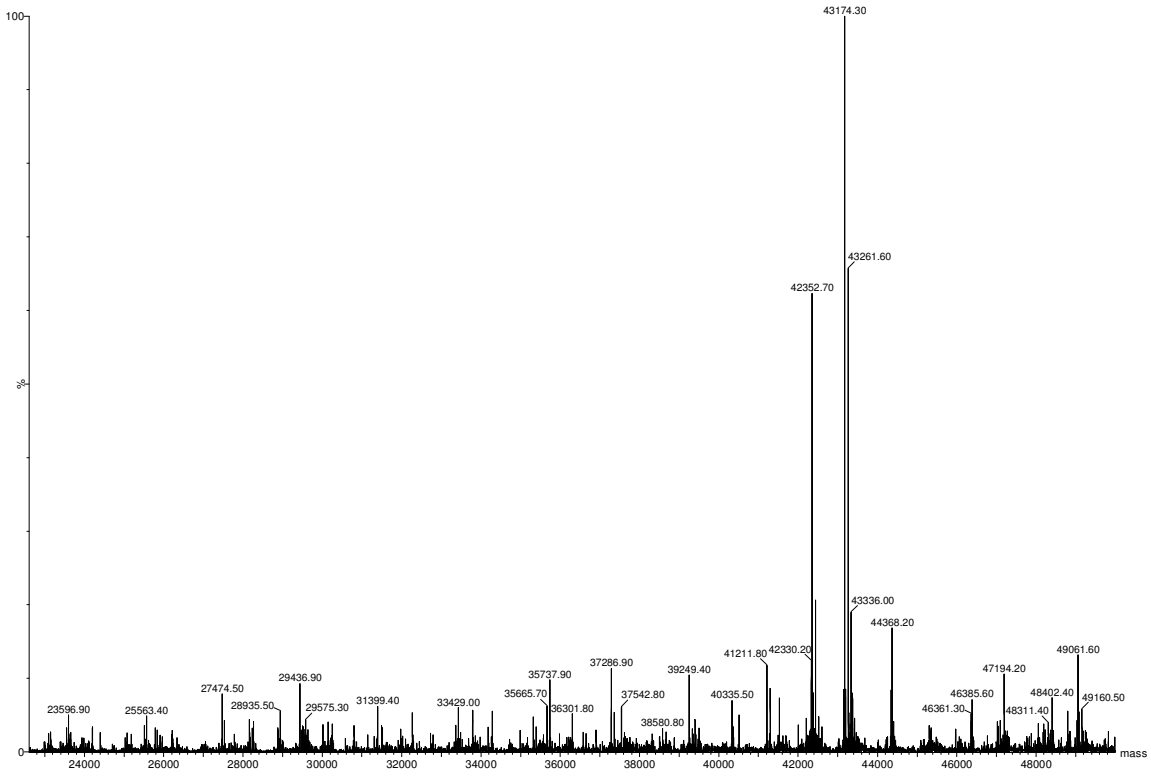

Figure 8.3. Full mass spectrum of HRP:melamine (1 µmol:15 µmol) by LCMS analysis. Separation was carried out on a C_{18} column with an acetonitrile gradient containing 0.1 % formic acid and detected by ESI-mass spectrometer in positive mode. The molecular mass of the major species is 43 174.30.

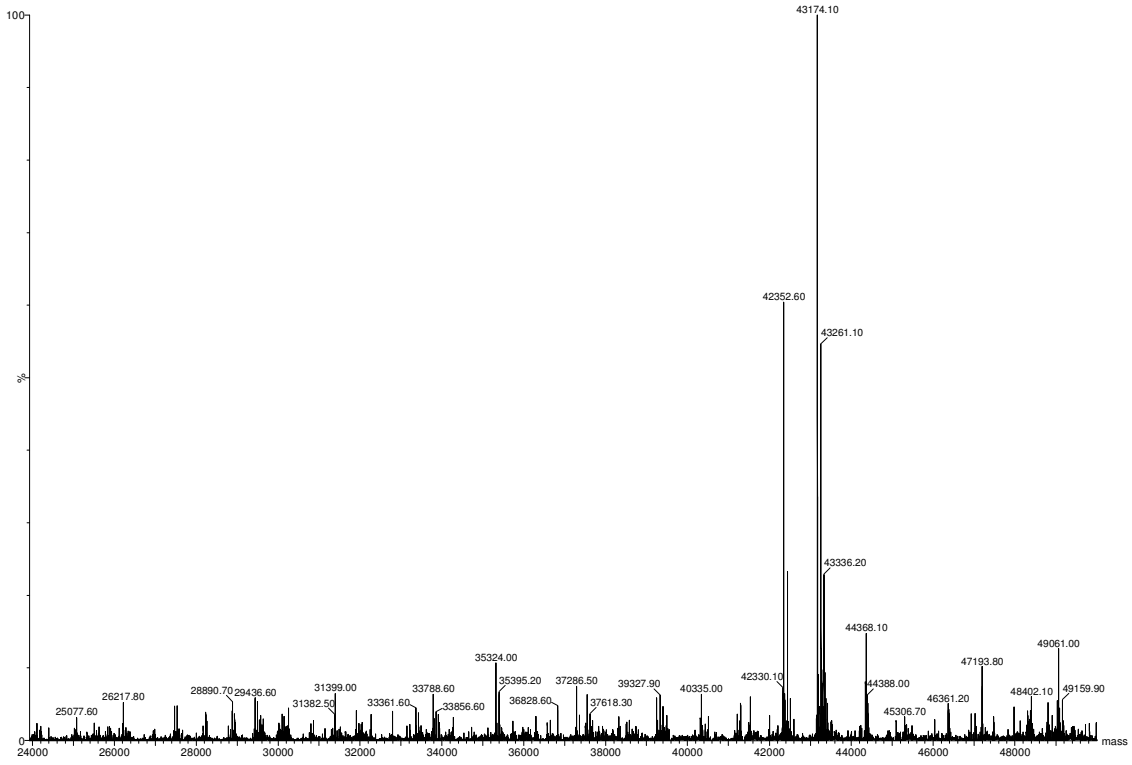

Figure 8.4. Full mass spectrum of HRP:melamine (1 µmol:30 µmol) by LCMS analysis. Separation was carried out on a C_{18} column with an acetonitrile gradient containing 0.1 % formic acid and detected by ESI-mass spectrometer in positive mode. The molecular mass of the major species is 43 174.10.

8.3 Coupling by reaction of an amine and aldehyde following oxidation by periodate

The first step in this coupling method is the oxidation of carbohydrates on the enzyme surface. HRP-C purified in this study (0.2 mg) was dissolved in water and carbohydrates were oxidized by adding 0.1 M sodium *meta*-periodate in 10 mM sodium phosphate buffer (pH 7.0) to the HRP solution (final concentration of 3 mg/ml). The solution was incubated at room temperature for 20 minutes. After incubation the solution was dialyzed overnight against 1 mM sodium acetate (pH 4.0) at 4˚C. The dialysate was removed from the dialysis tubing and freeze-dried. HRP-C as well as the oxidized sample were analysed by infra-red spectroscopy (figure 8.5 and 8.6).

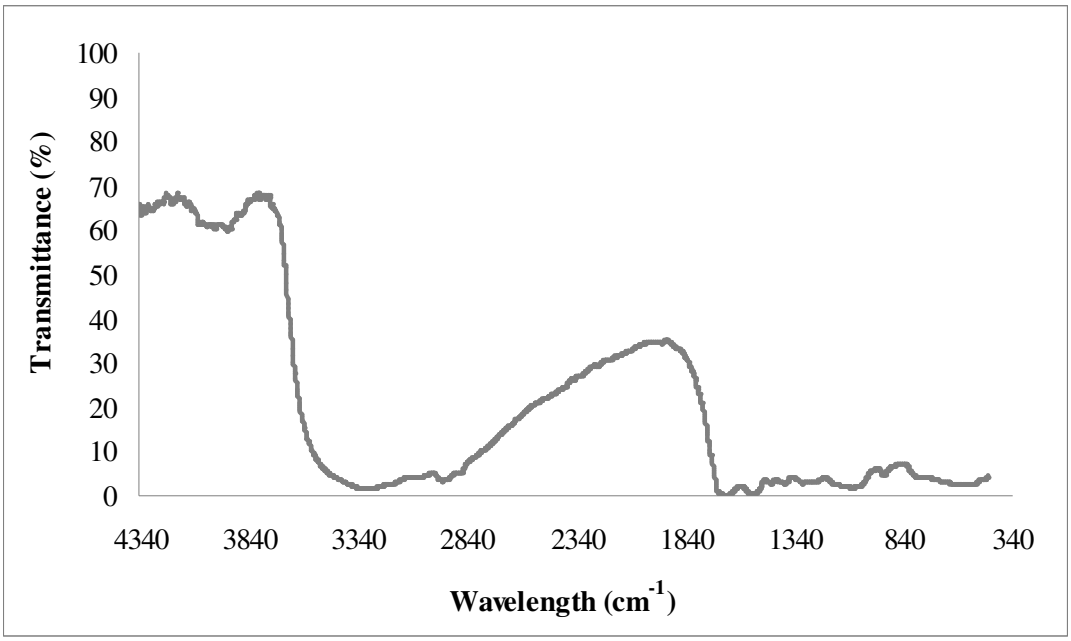

Figure 8.5. Infra-red spectra of HRP-C prior to oxidation.

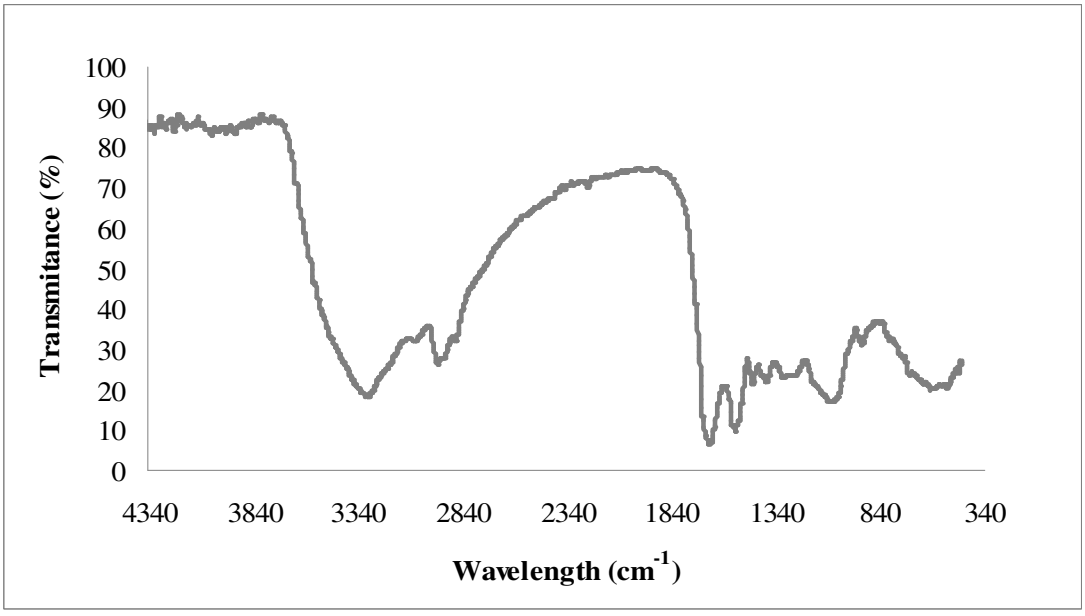

Figure 8.6. Infra-red spectra of oxidized HRP-C. Aldehydes were detected in the range of 1740-1680 cm^{-1}.

The oxidation protocol was repeated: 5 mg HRP-C was dissolved in 1.2 ml water and added to 0.3 ml 0.1 M sodium *meta*-periodate in 10 mM sodium phosphate buffer (pH 7.0). The solution was incubated at room temperature for 20 minutes and then dialyzed overnight against 1 mM sodium acetate (pH 4.0) at 4˚C. The dialysate was removed from the dialysis tubing and added to 0.5 ml melamine solution (10 mg/ml in 20 mM sodium carbonate buffer, pH 9.5). The HRP:melamine mixture was incubated at room temperature for 2 hours. After incubation, 100 µl of sodium borohydride (4 mg/ml in water) was added to reduce Schiff bases that might have formed and the solution was incubated at 4˚C for 2 hours. The solution was then dialyzed overnight against several changes of PBS at 4˚C. After dialysis the solution was removed from the dialysis tubing and freeze-dried for analysis by LCMS. The freeze-dried sample was, however, insoluble in water. The protocol was repeated and the final dialysate was analyzed by LCMS but could not be resolved by the LCMS gradient system used for HRP in this study.

8.4 Discussion

This study proposed a solid-phase immunobinding assay based on two-site ELISA techniques for the quantification of melamine in samples. The first step in the development of this assay was the production of a melamine-HRP conjugate. Two methods for coupling of melamine to HRP were investigated.

First the coupling of melamine to by amide bond formation between an amine group of melamine and carboxylic acids of the enzyme was tested. A carbodiimide was used to activate the carboxylic acids prior to incubation with melamine. LCMS analysis showed no significant additions to the molecular mass of HRP-C and the major species in both HRP:melamine samples corresponded with the control enzyme unbound to melamine (figures 8.2-8.4). It therefore appears that the yield of HRP-melamine conjugate produced by this reaction was too low to detect or that coupling was unsuccessful. Although many studies have successfully coupled HRP to antibodies using bifunctional reagents such as carbodiimides, these reactions are known to produce low yields of coupled HRP [131]. A possible explanation for the low coupling efficiency is the blockage of the carboxylic acids of HRP either by the surface carbohydrates or the three-dimensional shape of the enzyme itself. Other coupling studies generally include linker molecules for the coupling of melamine to carboxylic acids, suggesting that the amine groups of melamine do not readily react with carboxylic acids [132].

As discussed in chapter 2, carbohydrates account for approximately 18% of the molecular mass of HRP. Since the carbohydrates are not required for enzymatic activity, it was proposed that oxidation of the carbohydrates might be used for the formation of aldehyde groups which, in turn, can react with the amine groups of melamine to form an HRP-melamine conjugate. Infra-red spectra of both HRP-C and oxidized HRP-C show transmittance in the range of aldehydes (1740-1680 cm^{-1}) and notable changes can be seem in the spectra after oxidation. This suggests that oxidation of the enzyme was successful. The insolubility of the freeze-dried HRP:melamine sample and the inability to analyze the liquid sample by LCMS could be explained by the formation of aggregates due to self-coupling of the oxidized enzyme. HRP contains amino acid residues with α- and ϵ-amino groups (see table 2.3) and it is possible that these amino groups react with the aldehyde groups formed by oxidation of the carbohydrates. Studies have shown that treatment with fluorodinitrobenzene (FDNB) prior to oxidation prevents self-coupling [131]. In future, the addition of a FDNB treatment step in the production of an HRP-melamine conjugate should be considered.

Development of the proposed melamine-detection immunoassay is dependant on successful coupling of melamine to HRP without the loss of enzymatic activity. The results obtained in this study suggest that oxidation of surface carbohydrates followed by incubation with melamine could produce such a conjugate. Future developments of this immunoassay should also include the production of melamine-specific antibodies and development of a standard melamine dilution series for calibration.

9. DISCUSSION

HRP is a heme-containing glycoprotein primarily expressed in horseradish roots [1]. The enzyme exists as a family of distinctive isoenzymes which are divided into acid, neutral or basic groups based on respective isoelectric points [2]. Neutral isoenzyme C, the most abundant form of HRP, has been studied extensively and is used in most commercial applications of the enzyme. It is a single polypeptide chain and contains a heme group, two calcium-binding centres as well as branched heptasaccharides on the enzyme surface [4]. The cyclic reduction and oxidation of the heme group allows HRP to oxidize a variety of substrates [21]. Although specific *in vivo* functions have not been assigned to individual isoenzymes, it appears that HRP plays a role in plant hormone metabolism and structural defence mechanisms [4].

HRP-C has been the focus of most studies while the other isoenzyme groups have not been completely characterized. This has contributed to the current inability to distinguish and quantify the isoenzyme content of raw material during initial purification processes. This study investigated possible methods for isoenzyme quantification. For this purpose, HRP-isoenzymes were isolated and purified by adapted industrial processes. Through repeated salt fractionation steps and cation-exchange chromatography two forms of HRP were isolated. Although the molecular mass for both forms, as determined by SDS-PAGE, was similar, more detailed LCMS analysis revealed that the major species present in the first isolate corresponds with the molecular mass of an HRP-C standard (43 178 and 43 174.50, respectively). The molecular mass of the second isolate (42 356.1) was 822 less than the HRP-C standard. This difference in molecular mass could be accounted for by a carbohydrate group and it is therefore possible that the second isolated was another neutral isoenzyme or a less glycosylated form of HRP-C. The purity of the isolated isoenzymes was sufficient for the purpose of the immunological investigations proposed in this study. The first and second isolate were subsequently termed HRP-C and HRP-B.

Purified HRP-C, as well as other forms and components of HRP, were characterized by two HPLC systems. Absorbance was detected at 280 nm and 403 nm (specific for proteins and heme, respectively). The first HPLC analysis used an established salt gradient elution system and a cation-exchange column. The elution profiles of purified HRP-C and the HRP-C standard both contain a single peak with retention time of 16 minutes, detected at 280 nm and 403 nm. Analysis of a crude HRP preparation revealed a peak corresponding to HRP-C, in addition to other smaller protein peaks. Integration methods could be used to quantify each peak and therefore to determine the relative HRP-C content of a crude preparation. Due to the sensitivity of the cation-exchange column

for impurities in the matrix solution, this HPLC system cannot be used to analyse primary root extracts and does therefore not solve the inability to determine isoenzyme content prior to purifications steps.

Reversed-phase HPLC is a more robust analysis method and was investigated as an alternative isoenzyme-detection method due to the constraints of cation-exchange HPLC. A gradient elution system was successfully developed for reversed-phase HPLC analysis of HRP.

A reversed-phase HPLC system, using a C_{18} column, was also developed to characterize HRP. Elution profiles of the HRP-C standard and purified HRP-C showed a similar heme-containing peak with a retention time of 11 minutes. Apo-HRP was also characterized on the same system and revealed a single peak at 2 minutes, measurable only at 280 nm. Small amounts of apo-HRP could be detected in the elution profiles of the HRP-C standard, purified HRP-C and a crude HRP preparation. From cation-exchange and reversed-phase HPLC analyses of apo-HRP it was evident that removal of the heme group changed the ionic and hydrophobic characteristics of the enzyme significantly. The reversed-phase HPLC system developed in this study is a more robust analysis method compared to the cation-exchange HPLC system and could therefore be used to analyse crude samples and primary root extracts. For further development of cation-exchange and reversed-phased HPLC as isoenzyme quantification methods other isoenzymes need to be purified and characterized.

Other possible isoenzyme quantification methods, based on immunological techniques, were also investigated. It was hypothesized that electrophoresis followed by western blot analysis could be used to resolve and quantify isoenzymes in primary root extracts. Purified HRP-B and HRP-C were used to produce polyclonal antibodies for these immunological investigations. ELISA and western blot analysis revealed a degree of cross-reactivity for both anti-HRP-B and anti-HRP-C. The cross-reactivity of the antibodies could possibly be attributed to identical antigen determinants. Since no difference in molecular mass could be detected between HRP-B and HRP-C by SDS-PAGE, antibodies that are specific for the respective isoenzymes are essential for the detection and subsequent quantification of isoenzymes in raw material. Further investigations should therefore consider the production of monoclonal antibodies against different HRP-isoenzymes. While polyclonal antibodyies contain a heterogenous mixture of antibody structures, monoclonal antibodies are chemically defined, single-specificity molecules that are able to react with a single antigen determininant. It is important to note that, although monoclonal antibodies offer increased specifity, homogeneity and reproduciblity, the average affinity of monoclonal antibodies for the

antigen is generally lower than that of polyclonal antibodies. Production of monoclonal antibodies is also more time-consuming. However, unless antibodies specific for single isoenzymes can be produced, SDS-PAGE combined with western blot analysis cannot be used for isoenzyme quantification.

Instead of using gel electrophoresis as a separation method it was subsequently decided to analyze the different isoenzymes of HRP using a combination of isoelectric focusing followed by western blot analyses. Isoelectric focusing on pre-cast gels (pH gradient 3-10) revealed two bands for both purified HRP-C and the HRP-C standard. The bands focused at lower pH-values (7.2 and 7.8) than the reported pI range of 8.2-9.6 [4]. This difference could, however, be attributed to discrepancies in experimental conditions of pI determination studies. Both bands could also be detected by western blot analyses. It therefore appeared that these samples contained at least two forms of HRP. Subsequently, isoelectric focusing of a crude HRP preparation, followed by western blot analyses revealed two definite bands which correspond to those of HRP-C. Isoelectric focusing followed by western blot analysis could therefore be used to investigate the HRP-content of a sample. Isolation of other HRP-isoenzymes and subsequent characterization by a standard isoelectric focusing protocol is needed to further develop this method for isoenzyme quantification. Isoelectric focusing is, however, a labour-intensive technique that requires specialized equipment and is not, therefore, a practical solution to isoenzyme quantification in industrial production of HRP.

The final part of this study investigated the formation of an HRP-melamine conjugate by means of two chemical reactions. The production of an HRP-melamine conjugate with intact enzyme activity is a crucial component in a melamine quantification immunoassay currently being developed in our laboratory. The first coupling reaction, based on amide bond formation between an amine group of melamine and carboxylic acids of HRP in the presence of an activating carbodiimide, showed no significant formation of an HRP-melamine conjugate. This can be attributed to the low reactivity of the melamine amine groups with carboxylic acids or possibly explained by blockage of the carboxylic acids of HRP by its three-dimensional structure.

The second coupling reaction was based on the premise that the amine groups of melamine react more readily with aldehyde groups than with carboxylic acids. Aldehyde groups can be formed by the oxidation of the surface carbohydrates. Infra-red spectra of oxidized HRP-C showed transmittance in the range of aldehydes (1740-1680 cm^{-1}). Subsequent addition of melamine to oxidized HRP produced an insoluble sample that could not be resolved using LCMS. It is possible that oxidation of HRP leads to the formation of aggregates due to self-coupling by newly-formed

aldehyde groups and amine groups of certain amino acid residues. Further investigations should include a step to block amine groups of HRP prior to oxidation of carbohydrates.

While our current knowledge of HRP-C is sufficient for the development of commercial applications such as immunoassays, characterization of other isoenzymes remains crucial to the development of isoenzyme quantification methods. Future work should therefore include sequencing and crystal structure determination of acid and basic isoenzyme groups as well as comparisons of carbohydrate content within these groups.

10. EXPERIMENTAL METHODS

10.1 Isolation and purification of HRP from horseradish roots

One kg frozen horseradish roots was cut into cubes and homogenized in three volumes of 0.4 M NaCl in a Waring Blender. The homogenate, or primary root extract, was stirred for 1 hour at 20˚C. Subsequently, the primary root extract was filtered through 100 micron nylon mesh, the solid waste discarded and the resultant filtrate clarified through a Hyflo filter-aid 545 bed on Buchner filter paper under negative pressure. The final volume of this clarified extract was 3 070 ml.

10.1.1 First ammonium sulphate precipitation

All ammonium sulphate fractionation steps were carried out at 20˚C. Solid ammonium sulphate was added to the filtrate (see section 10.1) to 40% saturation and the pH adjusted to 5.0. The precipitate was allowed to settle before the supernatant was clarified through a Hyflo filter-aid 545 bed. The clarified supernatant (3 320 ml) was then brought to 85% ammonium sulphate saturation. The solution was again filtered through a Hyflo filter-aid 545 bed. The precipitate, together with the filter-aid, was collected from the filter and dissolved in a minimal volume of deionised water. This solution was filtered through filter paper and washed until the wash was clear. The filtrate was subsequently brought to 75% ammonium sulphate saturation and allowed to precipitate. The solution was filtered through Viking filter paper and the precipitate, collected from the filter paper, was dissolved in a minimal volume of deionised water. The dissolved precipitate was dialyzed overnight in dialysis tubing (14 000 MW cut off) against 20 ℓ deionised water.

10.1.2 First CM-Sepharose chromatography

The dialysate (116 ml) was clarified through a Hyflo filter-aid 545 bed before being transferred to a CM-Sepharose column (30 x 270 mm), previously equilibrated with 5 mM sodium acetate (pH 4.5). The resin was washed with one volume of equilibration buffer before eluting with 50 mM sodium acetate (pH 4.5) at a flow rate of 2 ml/min. Absorbance was detected at 280 nm and fractions containing HRP were identified from the elution profile after HRP activity assays (section 10.5.1). HRP-containing fractions were combined and subjected to cation-exchange HPLC.

10.1.3 Second ammonium sulphate precipitation

The combined fractions (55 ml) were brought to 75% ammonium sulphate saturation and allowed to precipitate overnight. The precipitate was dissolved in deionised water and subsequently dialyzed overnight in dialysis tubing (14 000 MW cut off) against 20 ℓ deionised water.

10.1.4 Second CM-Sepharose chromatography

The dialysate (18 ml) was transferred to a CM-Sepharose column (10 x 50 mm) previously equilibrated with 5 mM sodium acetate (pH 4.5). The resin was washed with one volume of equilibration buffer before eluting with 50 mM sodium acetate (pH 4.5) at a flow rate of 1 ml/min. Absorbance was detected at 280 nm and fractions containing HRP were identified from the elution profile after HRP activity assays. HRP-containing fractions were combined and subjected to cation-exchange HPLC analysis.

10.2 Preparation of apo-HRP from crude HRP

Crude HRP (3.5 g), provided by BBI Enzymes, was dissolved in 140 ml cold water and the pH of the solution was adjusted to 2 with 1 M HCl. Acetone was slowly added to the solution while stirring at 20ºC until a white precipitate formed. The solution was then stirred at 4ºC for an additional 30 min followed by centrifugation at 4592 *g* for 13 min at 0ºC using a Beckman JA-17 rotor. The supernatant was discarded and the pellet re-suspended in deionised water. The acidified acetone precipitation steps and centrifugation was repeated 4 times. The final resulting pellet was resuspended in deionised water and dialyzed overnight in dialysis tubing (14 000 MW cut off) against 5 ℓ deionized water. The dialysate was freeze-dried after which the protein content and enzymatic activity was determined (section 10.5.1). Apo-HRP was subjected to cation-exchange and reversed-phase HPLC analyses.

10.3 Isolation and purification of isoenzymes from crude HRP

Crude HRP (1.5 g), provided by BBI Enzymes, was dissolved in 15 ml deionised water and dialyzed overnight in dialysis tubing (7 500 MW cut off) against 10 ℓ deionised water.

10.3.1 First CM-Sepharose chromatography

The dialysate was filtered using Millipore Millex-HV hydrophylic polyvinylidene difluoride syringe-tip filters (0.45 μm pore size). The filtered dialysate (26 ml) was then transferred to a CM-Sepharose column (30 x 270 mm) previously equilibrated with 5 mM sodium acetate (pH 4.4). The resin was washed with equilibration buffer until non-adsorbed proteins had eluted as detected by absorbance at 280 nm. The protein was then eluted from the column with a linear gradient consisting of equal volumes 5 mM sodium acetate (pH 4.4) and 50 mM sodium acetate (pH 4.4). A linear flow rate of 9.76 cm/h was maintained.

Fractions containing peak B were identified from the elution profile (figure 5.4) and combined. This combined fraction, fraction B, was dialyzed overnight in dialysis tubing (14 000 MW cut off) against deionised water. The fraction B dialysate was freeze-dried (192 mg) and the protein content, enzymatic activity and RZ determined. Fractions containing peak A were identified from the elution profile and combined (fraction A).

10.3.2 Second CM-Sepharose chromatography

Fraction A (148 ml) was transferred to a CM-Sepharose column (3 x 27 cm) previously equilibrated with 5 mM Tris-HCl (pH 8.0). The resin was washed with equilibration buffer until non-absorbed isoenzymes eluted as detected through absorbance at 280 nm. The column was then eluted with 10 mM sodium carbonate (pH 9.6) at a flow rate of 2 ml/min. Fractions containing HRP were identified from the elution profile after HRP activity assays (see section 10.5.1). These fractions were combined and dialyzed overnight in dialysis tubing (14 000 MW cut off) against 20 ℓ deioinised water. The dialysate was freeze-dried (276 mg) and the protein content, enzymatic activity and RZ determined. Fraction A was subjected to cation-exchange and reversed-phase HPLC analyses.

10.4 Protein determination

Protein standards ranging from 0 to 2 mg/ml with 0.4 mg intervals were prepared using bicinchoninic acid (BCA) protein assay kits (Pierce 23227). Bovine serum albumin (2 mg/ml) was used as a protein standard and a standard curve was plotted from which unknown protein concentrations were determined.

10.5 Spectrophotometric analyses of HRP

10.5.1 Guaiacol assay

Peroxidase activity was measured according to the method of Bergmeyer [11] using guaiacol as hydrogen donor. The enzymatic activity was determined by the rate of H_2O_2 decomposition by HRP, measured spectrophotometrically as the rate of colour development at 436 nm. One unit of enzyme activity (U) is equal to an absorbance change of 0.001 per minute at 25°C and pH 7.0.

This method was used to measure the peroxidase activity of purified isoenzymes as well as that of samples from various purification steps in the isolation of HRP from roots. A sample from a specific purification step (100 µl) or 100 µl isoenzyme dissolved in assay buffer (0.1 M potassium phosphate buffer, pH 7) was added to the following mixture:

- 2.8 ml assay buffer (at 25˚C)
- 0.05 ml 0.018 M guaiacol (at 4˚C)
- 0.05 ml 0.025% H_2O_2 (at 4˚C).

Samples were diluted with assay buffer (20˚C) until ΔAbs/min fell within 0.040 – 0.045 range.

10.5.2 RZ determination

The absorbance of samples from purification steps during the isolation and purification of crude HRP as well as HRP-isoenzymes was recorded at 403 nm and 280 nm using UV-VIS spectrophotometry. A solution of the same composition as the sample being measured was used as a reference. Dilutions were made of the samples with the respective reference solutions until absorbance at 280 nm did not exceed 0.4 and absorbance at 403 nm did not exceed 1.4. RZ is expressed as A_{403}/A_{280}.

10.6 Determination of the molecular mass of HRP by LCMS

For LCMS analysis all samples were dissolved in deionised water (detection limited to a minimum concentration of 1 mg/ml). Samples were separated on an Xbridge C_{18} column (2.1 x 50 mm) with a linear gradient consisting of eluent A (0.1% formic acid) and eluent B (acetonitrile) at 300 µl/min flow rate. Two µl sample was injected and the eluent of the column was directly introduced into an ESI-mass spectrometer (Waters API Q-TOF Ultima) in the positive mode.

10.7 Cation-exchange high performance liquid chromatography (HPLC)

Cation-exchange HPLC analysis was based on an industrial protocol and used a protein-Pak CM separation column (Waters WAT-039785). Absorbance was detected at 280 nm and 403 nm with a programmable multi-wavelength absorbance detector (Waters M-490). Chromatograms were analyzed using DELTA Chromatography Data System 5.5 software.

10.7.1 Eluents

Degassed nanograde solvents were used in eluent A (0.01 M acetate buffer, pH 4.4) and eluent B (0.01 M acetate buffer, pH 4.4, 0.2 M NaCl, pH 4.4). A gradient elution system was used for analysis of an HRP standard, the purified isoenzymes and crude HRP preparations (table 10.1). An isocratic system of 0.01 M acetate, 0.05 M NaCl (pH 4.4) on a CM ion-exchange column was used for the analysis of apo-HRP. A flow rate of 0.8 ml/min was maintained throughout both separation methods.

Table 10.1. Gradient elution system used in cation-exchange HPLC analysis of HRP.

Time (min)	Eluent flow rate (ml/min)	% Eluent A[a]	% Eluent B[b]
1	0.8	97	3
22	0.8	67	33
25	0.8	0	100
30	0.8	0	100
35	0.8	97	3

[a] 0.01 M acetate buffer, pH 4.4
[b] 0.01 M acetate buffer, pH 4.4, 0.2 M NaCl, pH 4.4

10.7.2 Sample preparation

The HRP standard, purified isoenzymes, crude HRP and apo-HRP were dissolved in eluent A used in the respective separation method.

10.8 Reverse phase high performance liquid chromatography (HPLC)

Reversed-phase HPLC analysis, based on previous studies [124], used a Waters Sentry Guard column, a Waters Symmetry300 ™ C_{18} 5.0 µm column (WAT-106154) and a gradient elution system of degassed nanograde solvents. Absorbance was detected at 280 nm and 403 nm using a programmable multi-wavelength detector (Waters M-490). Data was obtained using DELTA Chromatography Data System 5.5 software.

10.8.1 Eluents

The C_{18} column was equilibrated with 50% of eluent A (0.1% TFA) and 50% of eluent B (10% eluent A and 90% acetonitrile). Initially, four different eluent gradient systems were used to investigate the separation of HRP isoenzymes. The methods differed in the initial ratio of the two eluents and the gradients are summarized in table 10.2. The fourth method differed from the previous three: eluent A consisted of 50% of the original eluent A (0.1% TFA) plus 50% of eluent B (10% eluent A and 90% acetonitrile). Eluent B remained 10% TFA and 90% acetonitrile. For method 4 a linear gradient from eluent A to eluent B was run over 10 minutes. A flow rate of 1 ml/min was maintained throughout all four separation methods.

Table 10.2. Gradient elution systems of eluent A and eluent B used in initial reversed-phase HPLC analyses.

Method 1		**Method 2**		**Method 3**		**Method 4**	
Time (min)	%B	Time (min)	%B	Time (min)	%B	Time (min)	%B
0	30	0	50	0	50	0	0
1	30	1	50	1	50	10	100
11	100	11	100	9	100	15	100
14	100	14	100	10	100	20	0
22	30	22	50	18	50		
				20	50		

Separation method 1 was used for further HRP-analyses. The elution time of method 1 was adapted to allow column equilibration between sample injections (table 10.3).

Table 10.3. Adapted gradient elution system used in reversed-phase HPLC analyses.

Time (min)	Eluent flow rate (ml/min)	% Eluent A[a]	% Eluent B[b]
1	1	70	30
11	1	0	100
26	1	0	100
34	1	70	30

[a] 0.1% TFA in water
[b] 10% eluent A and 90% acetonitrile

10.8.2 Sample preparation

HRP samples (1 mg/ml) were dissolved in deionized water. Heme was dissolved in ethanol (1 mg/ml) and centrifuged. The supernatant was subsequently dissolved in 5 ml ethanol.

10.9 Sodium Dodecyl Sulphate Poly-acrylamide Gel Electrophoresis (SDS-PAGE)

10.9.1 Preparation of resolving gel (12% or 15%) and stacking gel (4%)

The SDS-PAGE-cell was assembled. For 12% gels 7 ml resolving gel buffer (0.375 M Tris-HCl, pH 8.8, 0.1% SDS m/v) was mixed with 3 ml resolving gel monomer stock (acrylamide 40% m/v, N,N-bismethylacrylamide 2.7% m/v). For 15% gels 2.5 ml resolving gel buffer (1.5 M Tris-HCl, pH 8.8) was mixed with 3.75 ml resolving gel monomer stock and 0.1 ml 10% SDS m/v. Ammonium peroxodisulphate solution (10% m/v) was freshly prepared for each gel. Regardless of % gel, 10 µl ammonium peroxodisulphate was added to the resolving gel mixture, followed by 10 µl N,N,N,N-tetramethylethylenediamine (TEMED). Gels were cast directly after the addition of TEMED. A small volume of distilled water was pipetted on top of the gel. The water was discarded after the gel had polymerized. For 4% stacking gel, 1.25 ml of stacking gel buffer (0.5 M Tris-HCl, pH 6.8), 0.05 ml 10% SDS m/v and 0.5 ml stacking gel monomer (acrylamide 40% m/v, N,N-bismethylacrylamide 2.7% m/v) were mixed. Freshly prepared ammonium peroxodisulphate (0.025 ml) and 0.005 ml TEMED were added to the mixture and the stacking gel was cast. A comb was inserted into the stacking gel before polymerization to form wells for sample application.

10.9.2 Preparation of samples

All samples were diluted with an equal volume sample buffer, 0.004% m/v bromophenol blue in 0.125 M Tris-HCl, pH 6.8, 4% m/v SDS, 20% v/v glycerol, 10% v/v 2-mercapto ethanol (Fluka 11337). The diluted samples were heated for 15 min at 90˚C and allowed to cool down to room temperature before being loaded onto the gel.

10.9.3 Gel electrophoresis of HRP

Electrophoresis was carried out using a Biorad Powerpac™ Basic system. The electrophoresis cell was filled with electrode buffer, 250 mM Tris, 192 mM glycine, 0.1 % m/v SDS (pH 8.3). After the proteins were loaded, a constant voltage of 100 V was applied until the front maker (bromophenol blue) reached the end of the resolving gel. After electrophoresis, gels were removed to be stained or used in immunoblotting experiments.

10.9.4 Staining and destaining procedures

Gels were stained overnight on a shaker in a Coomassie blue solution (0.125% Coomassie Brilliant Blue R-250, 50% v/v methanol, 10% v/v acetic acid). The staining solution was discarded and the gel destained in destain solution 1 (50% v/v methanol and 10% v/v acetic acid) for an hour and then in destain solution 2 (5% v/v methanol and 7% v/v acetic acid) overnight.

10.9.5 Determination of the molecular mass of purified HRP isoenzymes by SDS-PAGE

A 12% poly-acrylamide gel was prepared as described in section 10.9.1. The isolated HRP-containing fractions obtained from the CM-Sepharose column (fractions A and B), together with ten protein standards (Precision Plus Protein ™ Kaleidoscope ™ marker), were loaded on the gel. A lysozyme standard and HRP-C standard (provided by Roche) were also loaded on the gel. A constant voltage of 100 V was applied until the bromophenol blue reached the end of the running gel. The gel was subsequently removed to be stained and destained as described in section 10.9.4. The R_f values of the ten protein standards were calculated (table 10.4). A graph of R_f versus log Mr was constructed and the approximate molecular mass of the isolated fractions could subsequently be derived (figure 10.1).

Table 10.4. Determination of the molecular mass of HRP by SDS-PAGE.

Protein	Front (cm)	R_f	Log Mr	Mr
1	0.3	0.05	5.40	250000
2	0.6	0.11	5.18	150000
3	1.1	0.20	5.00	100000
4	1.4	0.25	4.85	70000
5	2.1	0.38	4.70	50000
6	2.7	0.49	4.57	37000
7	3.7	0.67	4.40	25000
8	4.1	0.75	4.30	20000
9	4.9	0.89	4.18	15000
10	5.2	0.95	4.00	10000

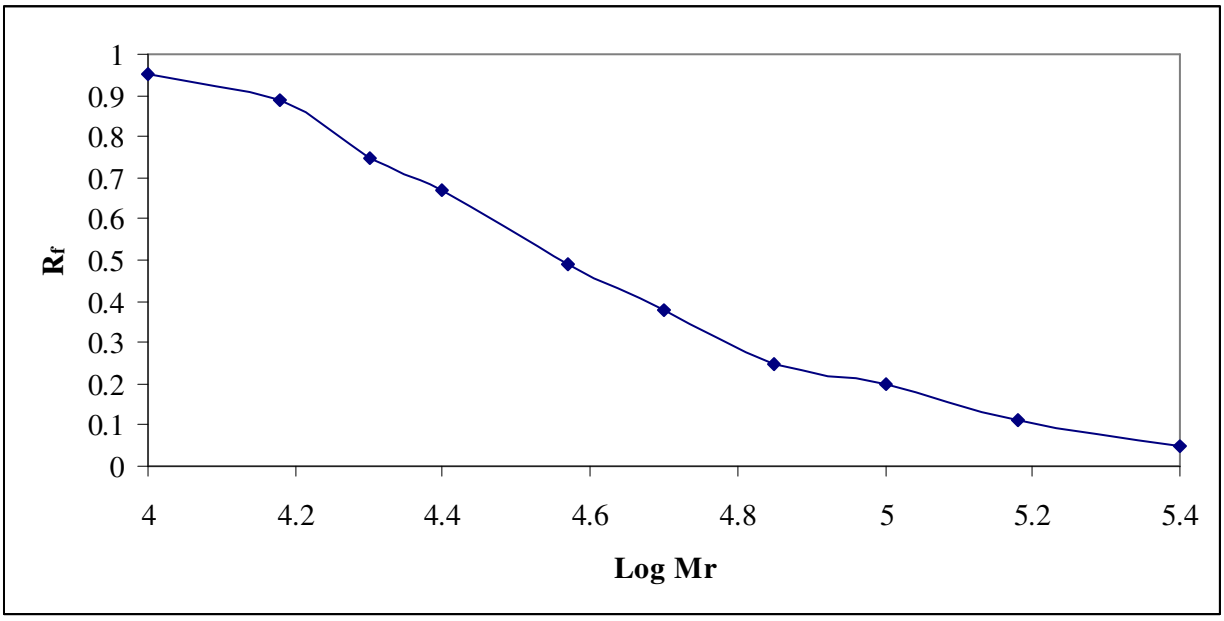

Figure 10.1. Determination of the molecular mass of purified HRP-isoenzymes by SDS-PAGE.

10.10 Isoelectric focusing (IEF)

HRP was resolved on 5% Servalyt™ PreNets™ using a horizontal electrofocusing system. The electrofocusing system consisted of a horizontal slab gel unit (HE-900), 1200 DC power supply (Hoefer Scientific Instruments) and a 220 V Hetofrig cooling system (Heto Birkerød).

10.10.1 Electrode solutions

Ten mm thick filter paper strips (1 cm in width) were cut to the width of the IEF gel and impregnated with either cathode solution (1 M NaOH) or anode solution (1 N phosphoric acid) before being placed on the gel.

10.10.2 IEF marker

A marker (Sigma I3018) containing a mixture of eight proteins with well-defined pI values ranging between 3.6 and 9.3 was used. The lyophilized marker was reconstituted with deionized water and aliquots were stored at -20˚C. Ten µl of marker solution containing ~36 µg of the protein mixture was loaded onto the IEF gel.

10.10.3 Sample preparation and application

HRP-isoenzymes were made up to different concentrations in deionized water. Samples were applied directly to the gel surface using silicone rubber applicator strips with 10 µl wells (Serva). Applicator strips were positioned in the centre of the gel and care was taken to ensure that the application strip was flat on the gel surface and free from air bubbles.

10.10.4 IEF conditions

Electrofocusing experiments were carried out at constant power (4 W). The current was set at a maximum value of 250 mA and voltage was set at a maximum value of 1200 V. Electrofocusing under these conditions would last between 2 – 4 hours.

10.10.5 Gel staining

After electrofocusing the applicator strip and electrode wicks were removed from the gel. For staining purposes the gel remained attached to the Gel-Fix™ support film. The gel was submerged in fixing solution (1.22 M tricholoracetic acid) for 10 minutes before being submerged in staining solution (4.5% Coomassie Blue R250, 45% ethanol, 10% acetic acid) for 5 minutes. The gel was destained for an hour in a solution containing 10% acetic acid and 30% ethanol and subsequently placed between transparencies to dry.

10.10.6 Determination of pI of HRP-C standard by IEF

A HRP-C standard was resolved on 5% Servalyt™ PreNets™ as described in sections 10.10.1-10.10.4. The pI marker, as well as different quantities of the HRP-C standard, was loaded on the gel shown in figure 8.4. After electrofocusing the gel was subsequently removed to be stained and destained as described in section 10.10.5. The R_f values of the eight protein standards were calculated (table 10.5). A graph of R_f versus pI was constructed and the approximate pI of the HRP-C standard could subsequently be derived (figure 10.2).

Table 10.5. Determination of the pI-value of an HRP-C standard with isoelectric electrofocusing.

Protein	Front	R_f	pI
1	1.4	0.13	3.6
2	2.8	0.27	4.6
3	3.6	0.34	5.1
4	4	0.38	5.9
5	5.1	0.49	6.6
6A	6.2	0.59	6.8
6B	7.4	0.70	7.2
7A	8.3	0.79	8.2
7B	8.7	0.83	8.6
7C	9.2	0.88	8.8
8	10.3	0.98	9.6

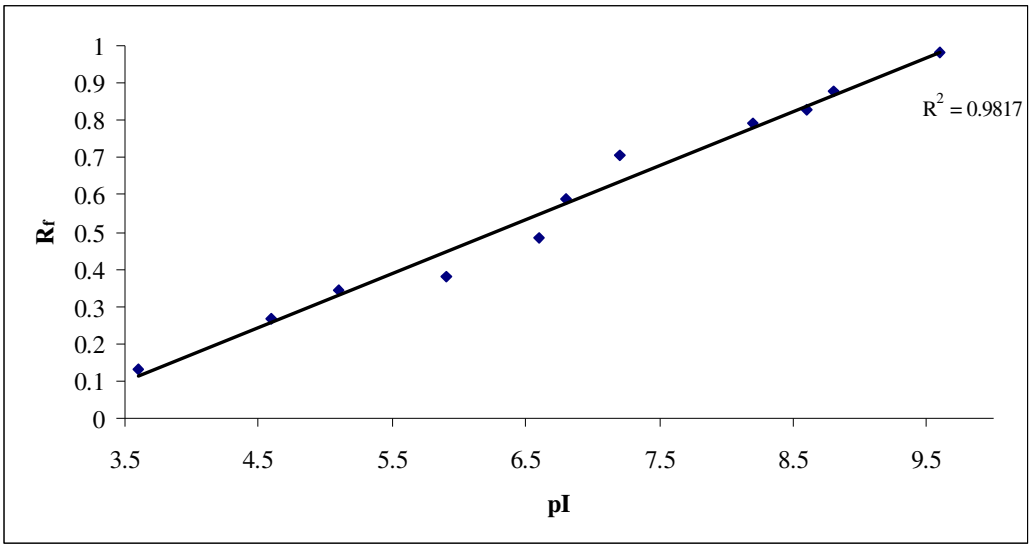

Figure 10.2. Determination of the pI-value of an HRP-C standard by isoelectric focusing.

10.10.7 Determination of pI of HRP-C and crude HRP by IEF

HRP-C purified in this study, together with crude HRP, were resolved by IEF as described in section 10.10.1-10.10.4. The pI marker, crude HRP, HRP-C and chymotrypsin, as an internal standard, were loaded on the gel. After electrofocusing, the gel was removed to be stained and destained as described in section 10.10.5. The R_f values of the eight protein standards were calculated (table 10.6). A graph of R_f versus pI was constructed and the approximate pI of the HRP-C standard could subsequently be derived (figure 10.3).

Table 10.6. Determination of the pI-value of HRP-C and crude HRP with isoelectric focusing.

Protein	Front	R_f	pI
1	1.4	0.14	3.6
2	2.2	0.21	4.6
3	3.1	0.30	5.1
4	3.8	0.37	5.9
5	4.8	0.47	6.6
6A	5.2	0.50	6.8
6B	6.3	0.61	7.2
7A	7.3	0.71	8.2
7B	7.6	0.74	8.6
7C	8	0.78	8.8
8	9.2	0.89	9.6

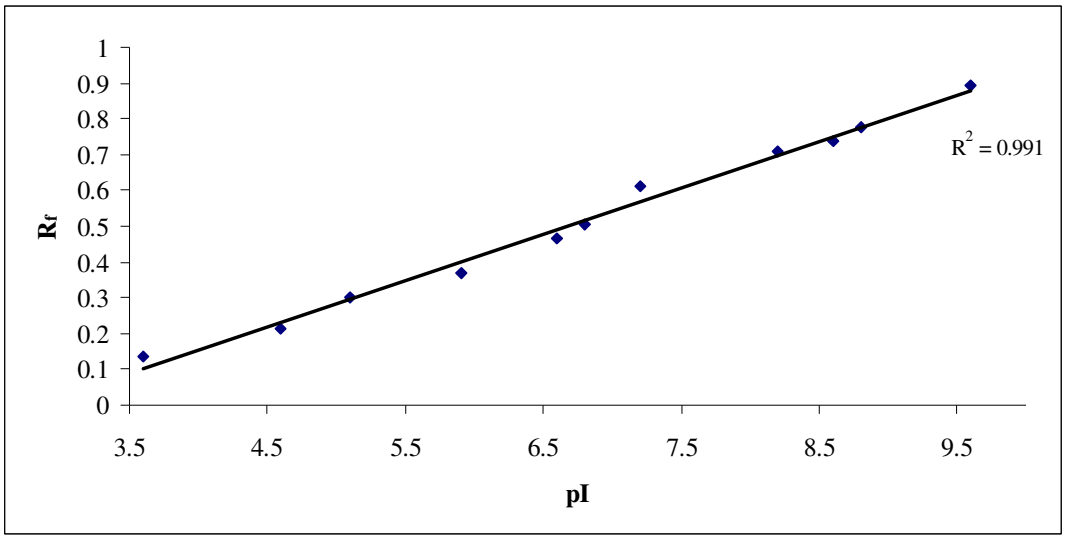

Figure 11.3. Determination of the pI-value of HRP-C and crude HRP by isoelectric focusing.

10.11 Preparation of antibodies against HRP-B and HRP-C

The production of antibodies was carried out using a method previously described by Bellstedt *et al.* [125].

10.11.1 Preparation of naked bacteria:protein complexes

A suspension of naked bacteria (1 mg/ml) was prepared in a small homogenizer. The NB suspension was subsequently mixed with either a HRP-B or HRP-C solution at a ratio of NB:isoenzyme (m/m) of 1:5. The NB:isoenzyme mixture was then dried on a rotary evaporator and the resulting precipitate resuspended in PBS by brief sonication to a final concentration of 240 µg NB:isoenzyme complex/0.5 ml PBS.

10.11.2 Immunization schedule

Rabbits were immunized with the NB:isoenzyme solution over a 49-day period. Blood was drawn on day 0 before the first immunization to use in experiments where pre-immune serum was needed. Rabbits were immunized with either 0.5 ml HRP-B or HRP-C solution on days 0, 3, 8, 14, 17, 21, 42, 45 and 49. Venous blood was collected on day 28 (2 ml) and day 56 (24 ml).

10.11.3 Antiserum preparation

Blood samples were incubated at 37˚C for 30 minutes to allow clotting. The clot was subsequently removed by centrifugation in a bench top centrifuge (800 x g). The supernatant (anti-serum) was aliquoted and stored at -20˚C.

10.12 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described previously [126]. Briefly, solutions of 0.05 mg/ml HRP-B or HRP-C were prepared in 0.05 M carbonate buffer, pH 9.6. Nunc Maxisorp microwell plates were coated with 100 µl of either HRP-B or HRP-C solution and incubated overnight at 4°C.

10.12.1 Inactivation of HRP with NaN_3

HRP-coated wells were incubated with 125 µl 0.1 M NaN_3 and 125 ul 0.1 M H_20_2 for 90 min at 37°C to inactivate the bound isoenzymes [69]. The wells were washed 10 times with PBS-Tween 20 solution.

10.12.2 Incubation steps

After the inactivation step the ligand-free surface of the microwells was saturated by incubation with casein buffer (10 mM Tris, pH 7.6, 0.15 mM NaCl, 0.5% Casein, 0.02% Thiomersol) at 37°C for 1 h. After saturation, the casein buffer was discarded and the wells were washed 3 times with PBS-Tween 20. Rabbit anti-HRP-B or rabbit anti-HRP-C in casein buffer containing Tween 20 was added and used to make dilution series of the respective primary antibody. The plates were incubated with 100 µl primary antibody per well for 60 min at 37°C. Washing was performed as mentioned above and 100 µl goat anti-rabbit HRP-conjugated secondary antibody (1:10 000 dilution in casein buffer containing Tween 20) was added per well. The plates were incubated for 60 min at 37°C, followed by 3 washes.

10.12.3 Substrate addition

One hunderd µl substrate solution (5 mg ABTS and 5 µl H_20_2 in 10 ml 0.1 M citrate buffer, pH 5.0) was added to each well and incubated at room temperature. Readings were taken 10, 15, 20 and 25 min after substrate addition at 405 nm using a Titertek Multiskan® Plus spectrophotometer. Absorbance readings were plotted against serum dilutions and the titer value for each antibody was calculated from the curve where the titer value is the serum dilution which gives an absorbance reading of 0.1.

10.13 Western blot analyses

10.13.1 Electro-transfer from SDS-PAGE

Proteins (purified HRP-B, HRP-C and a HRP-C standard) were separated on a 15% SDS-PAGE gel as described in section 10.9.1 and transferred to a nitrocellulose membrane using a Wet Transfer System (Biorad). Prior to electro-transfer, the nitrocellulose membrane was soaked in transfer buffer (50 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). The gel was placed on the nitrocellulose membrane and sandwiched between thick blotting paper and Scotch-Brite ™ pads. Electro-transfer was carried out at 100 V (constant voltage) for 70 minutes. After electro-transfer the membrane was blocked overnight at 4ºC with casein buffer (10 mM Tris, pH 7.6, 0.15 M NaCl, 0.5% casein, 0.02% Thiomersal).

10.13.2 Electro-transfer from IEF

An HRP-C standard, purified isoenzymes and crude HRP preparations were separated on 5% Servalyt™ PreNets™ as described in section 10.10.4 and transferred to a nitrocellulose membrane using a semi-dry transfer system. Prior to electro-transfer, the nitrocellulose membrane was soaked in transfer buffer (48 mM Tris, 39 mM glycine, 0.05% SDS, 20% methanol, pH 8.3). The gel was placed on the nitrocellulose membrane and the Gel-Fix™ support film was removed. The gel and membrane were sandwiched between layers of thick blotting paper soaked in semi-dry transfer buffer. Electro-transfer was carried out at 4 V for 60 minutes. After electro-transfer the membrane was blocked overnight at 4ºC with casein buffer (10 mM Tris, pH 7.6, 0.15 M NaCl, 0.5% casein, 0.02% Tiomersol).

10.13.3 Immunoblotting

Following protein transfer from either SDS-PAGE or IEF gel to a nitrocellulose membrane, the membrane was incubated overnight at 4ºC in casein buffer (10 mM Tris, pH 7.6, 0.15 M NaCl, 0.5% casein, 0.02% Thiomersal) to block non-specific binding to the membrane. The membrane was then incubated for 1 hour at 37ºC with an optimum dilution of either rabbit anti-HRP-B (5000x dilution in casein buffer) or rabbit anti-HRP-C (10 000x dilution in casein buffer). The membrane was washed 4 times for 15 minutes with PBS-Tween (0.15 M, pH 7.2 with 0.1% v/v Tween). The membrane was sequentially incubated for 1 hour at 37ºC with an optimum dilution goat anti-rabbit HRP-conjugated IgG (5 000x dilution in casein buffer), followed by washing with PBS-Tween as described before. The membranes were incubated with enhanced chemiluminescence detection reagents (Amersham ECL™ Western Blotting Detection Reagents) for 1 minute. ECL™ reagents were diluted 1:1 in 0.01M Tris-HCl, pH 8.0. The membrane was placed between transparencies, covered with X-ray film and placed in a hypercasette for exposure. Exposure times varied for different experiments.

10.14 Coupling of melamine and HRP

10.14.1 Coupling by reaction of an amine and carboxylic acid with EDC

HRP-C purified in this study was used to prepare a 20 mg/ml enzyme solution in 25 mM sodium phosphate buffer (pH 5.0). EDC solutions (8 mg/ml and 16 mg/ml) were also prepared in 25 mM sodium phosphate buffer (pH 5.0). Melamine solutions (2 mg/ml and 4 mg/ml) were prepared in 0.4 M sodium phosphate buffer (pH 8.0). Two reactions using different HRP:EDC:melamine ratios were evaluated (table 10.7).

Table 10.7. HRP:EDC:melamine ratios used in coupling reactions

Ratio ***(µmol)***	**HRP** ***(in 25 mM sodium phosphate buffer, pH 5)***	**EDC** ***(in 25 mM sodium phosphate buffer, pH 5)***	**Melamine** ***(in 0.4 M sodium phosphate buffer, pH 8)***
1:20:15	0.5 ml (20 mg/ml)	0.5 ml (8 mg/ml)	1 ml (2 mg/ml)
1:40:30	0.5 ml (20 mg/ml)	0.5 ml (16 mg/ml)	1 ml (4 mg/ml)

Equal volumes (0.5 ml) of HRP and EDC were added together and incubated at room temperature for 5 minutes. The HRP:EDC solution was then slowly added to 1 ml of the respective melamine solutions. The final solution (2 ml) was incubated overnight on a shaker at room temperature and subsequently transferred to a G10-Sephadex column (1 x 5 cm). The solution was allowed to elute under gravity. The resulting solution was freeze-dried and analyzed by LCMS.

10.14.2 Coupling by reaction of an amine and aldehyde following oxidation by periodate

Five mg of HRP-C purified in this study was dissolved in 1.2 ml water and added to 0.3 ml 0.1M sodium *meta*-periodate in 10 mM sodium phosphate buffer (pH 7.0). The solution was incubated at room temperature for 20 minutes and subsequently dialyzed overnight against 1 mM sodium acetate (pH 4.0) at 4˚C. The dialysate was removed from the dialysis tubing and 0.5 ml melamine solution (10 mg/ml in 20 mM sodium carbonate buffer, pH 9.5) was added. The HRP:melamine solution was incubated at room temperature for 2 hours. After incubation, 100 µl of sodium borohydride (4 mg/ml in water) was added and the solution was incubated at 4˚C for 2 hours. The solution was then dialyzed overnight against several changes of PBS at 4˚C. After dialysis the solution was removed from the dialysis tubing and freeze-dried.

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