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ASSAYS FOR PEROXIDASE ACTIVITY: THE HRP CASE

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ABSTRACT. Horseradish peroxidase (HRP) is a versatile oxidative enzyme with multiple practical applications. Furthermore, peroxidase activity is common in many other proteins, extending far beyond the class of peroxidases. As such, methods for assaying peroxidase activity have been sought for various applications. Here, a review of these methods is given, showing an inventory of almost 200 compounds employed as substrates in such assays, and with methods ranging from chronometric to colorimetric, fluorescent, polarography, or electron paramagnetic resonance (EPR) spectroscopy.

Keywords: *peroxidase, assay, phenols, HRP*

Horseradish peroxidase (HRP) is the archetypal heme peroxidase.[1-3] It is a globular glycoprotein with a mass of 42000, of which the protein moiety is approximately 34000, the rest of the molecular weight being accounted for by the prosthetic group (b-type heme), two calcium ions and some surface bound glycans.[4] The HRP structure (including a view of the active site) is shown in Figure 1. There are two similar, well-defined, domains within the apoprotein. Each domain contains one calcium ion, which provides structural stability and implicitly controls the enzyme's activity. The two domains provide a hydrophobic crevice in which the heme lies. A histidine occupies the fifth coordination position of the iron. This situation is very similar to that encountered in hemoglobin and myoglobin, so that the histidine is termed proximal histidine, and the protein domain providing this ligand is termed the proximal domain. The other domain is analogously named the distal domain and contains one relevant non-coordinated histidine, which is termed the distal histidine.[4]

The HRP general mechanism (Figure 2)[2,5,6] is initiated from the pentacoordinated ferric heme, binding peroxide. One of the H_2O_2 oxygen atoms then leaves as water, while the other is retained as a ferryl group to generate Compound I, featuring an Fe(IV) center coupled to a porphyrin cation radical.[7] Compound I then accepts one electron from a substrate molecule (typically an aromatic compound – phenolic or aminic), yielding Compound II, which still contains a ferryl group, but no porphyrin radical cation.[7]

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Compound II then accepts one electron from a second substrate molecule, yielding the enzyme native state (ferric). As to the fate of the substrate, loss of one electron, usually accompanied by loss of a proton, leads to the formation of a radical. The high reactivity and low selectivity usually associated with organic radicals make the chemistry of the peroxidase products particularly complicated.[4]

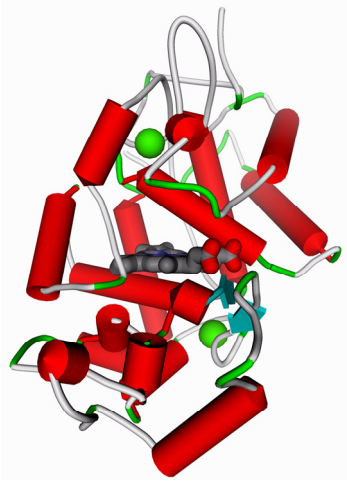

Figure 1. The HRP structure. The heme and the essential calcium ions are shown as sticks and spheres, respectively.

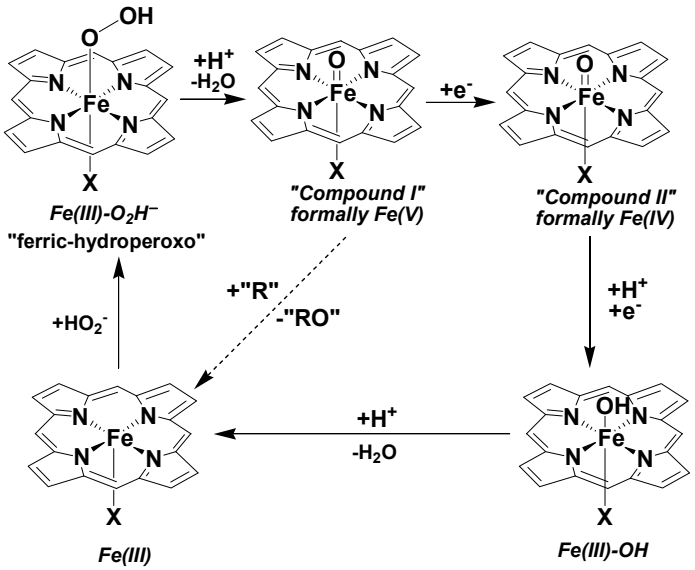

Figure 2. HRP general mechanism. Shown with a dashed arrow is the alternative peroxygenase reaction (R is typically an organic molecule).

Some substrates are oxidized by HRP in a single two-electron step: guaiacol (can be oxidized in either mono- or bi-electronic fashion), iodide, thyocyanate, and thioanisoles. There are also some (very few) instances where HRP acts as a peroxygenase, transferring an oxygen atom from the peroxide to the substrate (e.g., thioanisoles, hydroxylamine derivatives).[4,8]

One of the easiest ways to evaluate the HRP content of a preparation is by examination of its UV-VIS spectrum and determining the value of RZ (A_{403}/A_{280}, with 403 nm specific for heme and 280 nm specific for general protein material). For activity determinations, over 200 substrates, listed in Table 1, together with the type of method employed) have been used. These substances span most of the classes of substrates used by HRP, and employ several instrumental techniques – with UV-vis spectroscopy being the most widely-applied, as expected since many peroxidase substrates and products are colored.[4]

Table 1. Substrates and methods for HRP activity determination

Substrate	**Method**	**Observations**
ABTS (2,2'-azino-di[3-ethyl-benzothiazolin-(6)-sulfonate])	UV-vis[9-24]	the method of choice, also used in ELISA
pyrogallol	UV-vis[25-47]	(the result is also known as P.Z.-Purpurogallinzahl)
	fluorimetric[47-49]	
	polarographic[25]	
	titrimetric[25]	
	gasometric[25,50]	
guaiacol	UV-vis[9,17,18,51-64]	
	chronometric[65]	
	titrimetric[25]	
hydroquinone	polarographic[25,52,66-68]	the residual H_2O_2 or substrate concentration are determined
	fluorimetric[69]	the residual hydroquinone is detected with silver halides
	chronometric[70]	together with ascorbate
o-tolidine	UV-vis[23]	also in ELISA
5-aminosalicylic acid	UV-vis[15,23,64]	also in ELISA
o-phenylenediamine	UV-vis[15,22,23,25, 52,71-75]	also in ELISA; reducing agents such as sulfite stabilize the system by reacting with excess H_2O_2 after the enzymatic reaction has been terminated
	UV-vis[76,77]	in reversed micelles
	pH changes are measured[78]	
p-hydroxy-benzensulfonate	UV-vis[79]	coupled to 4-aminoantipyrine

Substrate	Method	Observations
4-aminoantipyrine	UV-vis[79]	coupled to p-hydroxybenzensulfonate
	UV-vis[80]	coupled to N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine
	UV-vis[21,23,57,81-85]	coupled to phenol; also in EIA (Trinder reagent) and also applied for model systems
	UV-vis[86]	coupled to N,N-diethyl-aniline
	UV-vis[87]	coupled to 2,4-diclorophenol
3,3',5,5'-tetramethyl-benzidine	UV-vis[12,15,23,52, 75,88-95]	also in ELISA; surfactants, cyclo-dextrin, penicillin (or derivatives thereof) may be used as additives
		after electrophoresis[96]
phenol	EPR[97]	
	UV-vis[21,23,85]	coupled to 4-aminoantipyrine, in EIA (Trinder reagent)
	UV-vis[52]	
5-phenyl-4-pentenyl-hydroperoxide (PPHP)	UV-vis[98]	selective peroxidase reagent, used together with a reducing substrate
fluorescein	fluorimetric[99,100]	
p-anisidine	UV-vis[46]	
3-methyl-2-benzothiazolinon hydrazone (MBTH)	UV-vis[79]	yields an indamine when reacting with m-dimethylaminobenzoic acid
	UV-vis[15]	ELISA
m-dimethyl-aminobenzoic acid	UV-vis[79]	yields an indamine when reacting with MBTH
luminol	fluorimetric[61,101-103]	in EIA; an impressive number of substances may be used as activators
L012 (luminol analog)	fluorimetric[104]	same as above
N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine	UV-vis[105]	coupled to 4-aminoantipyrine
3,3'-diamino-benzidine	UV-vis[82,106-109]	
p-phenylene-diamine	UV-vis[51,52,88,110]	
m-phenylene-diamine	UV-vis[52]	
pyrocatechin	UV-vis[25,52]	
	polarographic[25]	
	iodometric[12]	enzymatically produced quinone is titrated with iodine
o-dianisidine	UV-vis[88,110-116]	free or dextrane-bound
caffeic acid	fluorimetric[17]	
ferulic acid	fluorimetric[17]	
p-coumaric acid	fluorimetric[17]	
coniferyl alcohol	fluorimetric[17]	

Substrate	Method	Observations
5-(5'-azoluciferinyl)-2,3-dihydro-1,4-phtalazindione (ALPDO)	fluorimetric[117]	
p-hydroxy-phenylpropionic acid	fluorimetric[17,77,118-122]	also in ELISA; alone or together with a surfactant (CTAB or CTAC)
homovanillic acid	fluorimetric[123,124]	o-dianisidine may be used as activator
N,N-diethylaniline	UV-vis[77,86]	also coupled to 4-aminoantipyrine; also used for model systems
3-amino-9-ethylcarbazole	UV-vis[15]	ELISA
mesidine	UV-vis[9,25,125-128]	
ferrocyanide	[9]	
malachite green	UV-vis[25,41,52,129]	
guaiac resin	chronometric[65]	
	UV-vis[25]	guaiaconic acid is the main product
benzidine	chronometric[45]	ascorbate is also needed
	UV-vis[25,52,58,62, 130-135]	
2,6-dichlorophenol-indophenol	UV-vis[25,29,62,136,137]	
2,3',6-trichlorophenol-indophenol	UV-vis[25,138]	
pyrogallic acid	UV-vis[64,139]	yields gallopurpurin
phenolphtalein	UV-vis[25,52,64,140]	
o-toluidine	UV-vis[25,77,141]	
	chronometric[25,41]	ascorbate is also needed
guaiaconic acid	UV-vis[25]	
p-toluidine	UV-vis[25,77,141]	
acetylguaiacol	UV-vis[25]	
o-cresol	UV-vis[25,77,141]	
p-cresol	UV-vis[25,77,141]	
m-cresol	UV-vis[25,77,141]	
α-naphtol	UV-vis[25,142,143]	also coupled with dimethyl-phenylendiamine (NADI reagent) or with p-phenylendiamine (Guthrie reagent) to yield indophenols
aniline	UV-vis[52,77,144]	
ascorbate	UV-vis[25]	
	chronometric[25]	iodide, o-toluidine, or benzidine are used as second substrates
	polarographic[25]	
iodide	chronometric[25,145]	ascorbate is used as a second substrate
	chronometric[146]	starch, thiosulfate and iodide are also needed

Substrate	Method	Observations
	UV-vis or titrimetric[25]	
2,7-diaminofluorene	UV-vis[25]	
p-aminobenzoic acid	UV-vis[25,52]	
brilliant green	UV-vis[25]	
N,N-dimethyl-p-phenylene-diamine	UV-vis[52,147]	
thymol	UV-vis[52]	
resorcin	UV-vis[52]	
orcin	UV-vis[52]	
fluoroglucine	UV-vis[52]	
benzoic acid	UV-vis[52]	
salicylic acid	UV-vis[52]	
pyrocatechuic acid	UV-vis[52]	
galic acid	UV-vis[52]	
adrenaline	UV-vis[52]	
tyrosine	UV-vis[52]	
tryptophane	UV-vis[52]	
flavones	UV-vis[52]	
2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a]-pirazin-3-one	chemiluminescence[148]	luciferin analog
acetaminophen and its derivatives	fluorescence[120]	fluorescent 5,5'-diacetamido-2,2'-bisphenol formation
p-phenyl-phenol, p-cresol, 1-naphtol or aniline	fluorescence[149]	a second compound is used to trap HRP-generated substrate radicals
2,4-dichlorophenol	UV-vis[77,150]	also in ELISA
Lumigen PS-3	chemiluminescence[151]	western, southern
2-methyl-1-propenol	chemiluminescence[152]	coupled to a lipase
acridan-9-carboxylic derivatives	chemiluminescence[152]	
tyramide derivatives	fluorescence[153]	
homogentisic acid γ-lactone	chemiluminescence[154]	ELISA
3-(10-phenothiazinyl)-propanesulfone		employed together with luminol[155]
4-(4-hydroxy-phenylcarbamoyl)-butanoate	fluorimetric[156]	ELISA
acetanilide and its derivatives	fluorimetric[157]	
p-hydroxy-benzoic acid /terbium	chemiluminescence[158]	
alloxantin	chronometric[41]	ascorbate is also needed
1-amino-2-naphtol azo-derivatives	UV-vis[159]	

Substrate	**Method**	**Observations**
6-hydroxy-dopamine	UV-vis[160]	O_2 may oxidize the substrate, to yield H_2O_2; peroxidase accelerates the oxidation
eosine	chemiluminescence[161]	
4-methoxy-1-naphtol	UV-vis[162]	
4-and 5-substituted o-phenylendiamine derivatives	UV-vis[162]	
4-chloro-1-naphtol	UV-vis[162]	
2-hydroxy-2,4,6-cyclohepta-trienone (tropolone)	UV-vis[163]	coupled to 3-methyl-2-benzothiazolinone hydrazone (MBTH) or its derivatives
7 amino-indazole	UV-vis[164]	
5 amino-indole	UV-vis[164]	
7 amino-indole	UV-vis[164]	
5 amino-benzimidazole	UV-vis[164]	
7 amino-benzimidazole	UV-vis[164]	
5 amino-benzothiazole	UV-vis[164]	
7 amino-benzothiazole	UV-vis[164]	
5 amino-benzoxazole	UV-vis[164]	
7 amino-benzoxazole	UV-vis[164]	
5 amino-indazole	UV-vis[164]	
7-dimethylamino-naphtalen-1,2-dicarboxylic acid hydrazide	UV-vis[165]	
2-phenylamino-5-amino-pyridine	UV-vis[166]	
2-(4-hydroxy-3,5-dimethoxyphenyl)-4,5-bis(4-methoxyphenyl) imidazole,	UV-vis[167]	a phenolic derivative is used as mediator
2-(3,5-dibromo-4-hydroxyphenyl)-4,5-diphenylimidazole	UV-vis[167]	a phenolic derivative is used as mediator
2-(3-bromo-5-methoxy-4-hydroxyphenyl)-4,5-bis(4-methoxyphenyl) imidazole	UV-vis[167]	a phenolic derivative is used as mediator
4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxyphenyl) imidazole	UV-vis[167]	a phenolic derivative is used as mediator
4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxy-3-methoxy phenyl) imidazole	UV-vis[167]	a phenolic derivative is used as mediator
2-(4-hydroxyphenyl)-4,5-bis(4-methoxyphenyl) imidazole	UV-vis[167]	a phenolic derivative is used as mediator

Substrate	Method	Observations
4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxy-3,5-dimethoxyphenyl) imidazole	UV-vis[167]	a phenolic derivative is used as mediator
m-aminosalicylic acid	UV-vis[168]	
6-carboxy-3-methylbenzothiazolone-hydrazone	UV-vis[169]	coupled to aniline derivatives: N,N-(biscarboxymethyl)aniline; N,N-(biscarboxymethyl)-4-methoxyaniline; N,N-(bis-beta-carboxyethyl)aniline; coupled to aniline derivatives: N,N-(biscarboxymethyl)aniline; N,N-(biscarboxymethyl)-4-methoxyaniline; N,N-(bis-beta-carboxyethyl)aniline; N-ethyl-N-phenyl glicine; N-ethyl-N-carboxyethylaniline; N-phenylpiperidinyl succinate; N-ethylanilinopropanamine; N-methylanilinopropanamine; N-methyl-N-carboxyethylaniline; N,N-(bis-beta-carboxyethyl)2,5-dimethylaniline; N-β-carboxyethylaminobenzoate
N,N-disubstituted aniline derivatives; 4-amino-antipyrine, 4-(dimethylamino) antipyrine, 4-(ethylamino) antipyrine, 4-(methylamino) antipyrine, 4-(sulfonato-methylamino)antipyrine, 4-)sulfonatomethyl)(isobutyl) aminoantipyrine, 4-(sulfonato-methyl)(methyl)amino antipyrine, 4-(isopropyl)-amino antipyrine	UV-vis[170]	coupled to 3-methyl-2-benzotiazolinon hidrazone, 1-methyl-2-quinolin hidrazone, N-methyl-pyridon-4-hidrazone, N-methyl-pyridon-2-hidrazone, N-methyl-quinolin-2-hidrazone, N-methyl-quinolin-4-hidrazone, N-methyl-2-benzotiazolinon hidrazone, N-methyl-thiazolinon-2-hidrazone, N-methyl-thiazolinon-2-hidrazone, N-methyl-4-phenylthiazolinon-2-hidrazone, N-methyl-oxazolinon-2-hidrazone, N-methyl-benzoxazolinon-2-hidrazone, 1,3-dimethyl-benzimidazolinon-2-hidrazone, 3-(C1-4 alkyl)-2-benzotiazolinon hidrazone
methylen-bis (N-ethyl-N-sulfopropyl-3,5-dimethylaniline)	UV-vis[171]	
p-phenylendiamine derivatives	UV-vis[172]	
dicyanoethylaryl derivatives	UV-vis[173]	

Substrate	Method	Observations
3,3'-di-carbonyloxy- or sulfonyloxy-benzidine	UV-vis[174]	
3-amino-pyrazole derivatives	UV-vis[175]	
p-chlor-phenol	UV-vis[77]	
2,4-dibromophenol	UV-vis[77]	
p-bromophenol	UV-vis[77]	
2,3-dichlorophenol	UV-vis[77]	
2-nitrophenol	UV-vis[77]	
3-nitrophenol	UV-vis[77]	
2-aminophenol	UV-vis[77]	
m-aminophenol	UV-vis[77]	
2-bromoaniline	UV-vis[77]	
3-bromoaniline	UV-vis[77]	
2-chloroaniline	UV-vis[77]	
3-chloroaniline	UV-vis[77]	
m-toluidine	UV-vis[77]	
dimethylaniline	UV-vis[77]	
o-anisidine	UV-vis[77]	
m-anisidine	UV-vis[77]	
2-methyl-2,6-dinitrophenol	UV-vis[77]	
2-methoxy-5-nitroaniline	UV-vis[77]	
2-methyl-5-nitroaniline	UV-vis[77]	
3,5-dihydroxytoluene	UV-vis[77]	
3-methoxyphenol	UV-vis[77]	
2-amino-5-methylphenol	UV-vis[77]	
2-hydroxy-3-methylbenzoate	UV-vis[77]	
2-hydroxyphenylacetate	UV-vis[77]	
2,3-dimethylphenol	UV-vis[77]	
2,5-dimethylphenol	UV-vis[77]	
2-ethylphenol	UV-vis[77]	
3-ethylphenol	UV-vis[77]	
2-methoxymethylphenol	UV-vis[77]	
2,3-dimethylaniline	UV-vis[77]	
2,5-dimethylaniline	UV-vis[77]	
3,5-diethylaniline	UV-vis[77]	
3-(dimethylamino)-phenol	UV-vis[77]	
3-methoxy-N,N-dimethylaniline	UV-vis[77]	
N,N-diethyl-1,3-phenylendiamine	UV-vis[77]	
3,5-dimethyl-1,2-phenylendiamine	UV-vis[77]	
N-ethyl-N-(3-methylphenyl)-N'-acetylethylendiamine	UV-vis[77]	

Substrate	Method	Observations
fluorophenols	[176]	the liberated fluoride is quantitated
aromatic urea derivatives	UV-vis[177]	
2,4-dichloro-1-naphtol	UV-vis[178]	
phenoxazine derivatives	UV-vis[179]	
2-(2-phenyl-3-indolyl)-4,5-di[4-(dimethylamino)-phenyl]imidazole and similar imidazoles	UV-vis[180]	
4-methoxyphenol	UV-vis[181]	
4-ethoxyphenol	UV-vis[181]	
4-iodophenol	UV-vis[181]	
4-hydroxyphenyl-acetic acid	UV-vis[181]	
4-hydroxyhippuric acid	UV-vis[181]	
tyramine	UV-vis[181]	
chlorpromazine	UV-vis[182]	
(3-acylaminobenzo (b)furan-2(3H)one)	fluorimetric[183]	
hydroxycoumarines	fluorimetric[184]	
nitroxides	EPR[97]	hydroxylamines are formed
MeOOH	UV-vis[185]	in vivo estimation
H_2O_2	polarographic[25]	excess H_2O_2 is determined
	manometric[186]	

Besides the mentioned substrates, a very large number of compounds have been reported to be useful in the respective reaction mixtures (either as activators or stabilizers).[4]

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