RESEARCH COMMUNICATION

Oxidation of indole-3-acetic acid by dioxygen catalysed by plant peroxidases: specificity for the enzyme structure

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Indole-3-acetic acid (IAA) can be oxidized via two mechanisms: a conventional hydrogen-peroxide-dependent pathway, and one that is hydrogen-peroxide-independent and requires oxygen. It has been shown here for the first time that only plant peroxidases are able to catalyse the reaction of IAA oxidation with molecular oxygen. Cytochrome *c* peroxidase (CcP), fungal peroxidases (manganese-dependent peroxidase, lignin peroxidase and *Arthromyces ramosus* peroxidase) and microperoxidase were essentially inactive towards IAA in the absence of added H_2O_2. An analysis of amino acid sequences allowed five structurally similar fragments to be identified in auxin-binding proteins and plant peroxidases. The corresponding fragments in CcP and fungal peroxidases showed no similarity with auxin-binding proteins. Five structurally similar fragments form a subdomain including the catalytic centre and two residues highly conserved among 'classical' plant peroxidases only, namely His-40 and Trp-117. The subdomain identified above with the two residues might be responsible for the oxidation of the physiological substrate of classical plant peroxidases, IAA.

Key words: auxin-binding domain, auxin-binding proteins, peroxidase, structural similarity.

INTRODUCTION

Molecular mechanisms of the action of plant hormones remain a challenging problem of plant physiology and molecular biology. Plant peroxidases that are involved in stress- and pathogen-induced processes in plants [1] contribute towards molecular mechanisms of the plant immune response. Plant genomes contain numerous copies of peroxidase genes (up to 30 in *Arabidopsis* [2]), whose detailed functions are still unclear [3]. The changes in peroxidase expression patterns are interdependent on the level of endogenous auxins [4]. Application of exogenous auxins can induce [5] or suppress [6] peroxidase synthesis, and thus these switch either on or off peroxidase gene promoters. The whole set of physiological data indicates a key physiological role played by plant peroxidases; however, it is still difficult to determine which substrate is the physiologically relevant one for these enzymes.

Plant peroxidases (EC 1.11.1.7) catalyse the oxidation of numerous artificial and physiological electron donors (e.g. coniferyl alcohol, caffeic and ascorbic acid, etc.) utilizing hydrogen peroxide [7]:

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

$$EI + S \rightarrow EII + P \quad (2)$$

$$EII + S + H^+ \rightarrow E + H_2O + P \quad (3)$$

where E, EI and EII are the native enzyme and its compounds I and II respectively, and S and P are respectively the substrate and the product of its one-electron oxidation.

The only physiological reaction known in terms of its high specificity for the substrate is the oxidation of indole-3-acetic acid (IAA) by a hydrogen-peroxide-independent, oxygen-requiring route catalysed by plant peroxidases [8]. The reaction mechanism is extremely complicated due to overlapping radical processes [9,10]. The recent studies on the mechanism of IAA oxidation catalysed by horseradish and tobacco (*Nicotiana*) peroxidases [11–13] allowed us to identify skatolyl hydroperoxide as a key reaction product, and to conclude that there should be a special binding site for IAA in plant peroxidases. We proposed that plant peroxidases are highly specific IAA oxygenases, and the reaction cycle is initiated via formation of a ternary complex, enzyme–IAA–oxygen-yielding, IAA cation radical [11]:

$$E^{3+} + IAA \leftrightarrow [E\text{–}IAA] + O_2 \leftrightarrow [E\text{–}IAA - O_2] \quad (4)$$

$$[E\text{–}IAA\text{–}O_2] \leftrightarrow E + IAA^{\bullet +} + O_2^{\bullet -} \quad (5)$$

where $IAA^{\bullet +}$ is the IAA cation radical and $O_2^{\bullet -}$ is the superoxide anion radical.

Further decarboxylation yields skatolyl radicals [eqn. (6)], which react with oxygen [eqn. (7)] and, subsequently, form the corresponding organic hydroperoxide [eqn. (8)].

$$IAA^{\bullet +} \rightarrow InCH_2^{\bullet} + CO_2 \quad (6)$$

$$InCH_2^{\bullet} + O_2 \rightarrow InCH_2O_2^{\bullet} \quad (7)$$

$$InCH_2O_2^{\bullet} + IAA \rightarrow InCH_2OOH + IAA^{\bullet} \quad (8)$$

where $IAA^{\bullet}$ is the indolyl radical, and $InCH_2^{\bullet}$, $InCH_2O_2^{\bullet}$ and $InCH_2OOH$ are skatolyl radical, peroxy radical and hydroperoxide respectively.

The failure to detect compound I in the reaction course [12], and the necessity of an extra electron donor to complete the cycle [13], suggested similarities between the mechanism of action of plant peroxidases and prostaglandin H synthase [14]. The latter has the arachidonic-acid-binding site located approx. 12 Å (1.2 nm) away from the haem edge [15], i.e. we anticipated the

Abbreviations used: CcP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; LiP, lignin-dependent peroxidase from *Phanerochaete chrysosporium*.

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presence of a special binding site for IAA that was distinct from that involved with the binding and cleavage of hydrogen peroxide. This would permit IAA to bind on the enzyme simultaneously with indole-3-methanol, derived from skatolyl hydroperoxide [13]:

$$E + InCH_2OOH \rightarrow EI\text{-}InCH_2OH \rightarrow EII\text{-}[InCH_2OH]^{\cdot} \quad (9)$$

$$EII\text{-}[InCH_2OH]^{\cdot} + IAA \rightarrow IAA\text{-}EII\text{-}[InCH_2OH]^{\cdot} \rightarrow IAA^{\cdot}\text{-}EII\text{-}[InCH_2OH] \rightarrow IAA^{\cdot}\text{-}EII + InCH_2OH \quad (10)$$

The goal of the present study was to test and to examine all available haem-containing peroxidases in the reaction of IAA oxidation with molecular dioxygen to examine whether there is any similarity between haem-containing peroxidases and auxin-binding proteins. This approach has allowed us to show for the first time both the reaction specificity of the enzyme structure and the presence of structurally similar fragments in classical plant peroxidases and auxin-binding proteins.

MATERIALS AND METHODS

Chemicals

IAA, iron(II) chloride, microperoxidase MP-11, and haemin were purchased from Sigma, St. Louis, MO, U.S.A., indole-3-methanol and indole-3-aldehyde were from Aldrich, Milwaukee, WI, U.S.A., and hydrogen peroxide and its salts were from Merck, Darmstadt, Germany. Milli Q water was used for the preparation of all solutions.

Enzymes

Horseradish peroxidase (HRP) isoenzyme C was purchased from Biozyme Laboratories Ltd., San Diego, CA, U.S.A, and used without further purification. Soya-bean peroxidase, RZ 1.1 (where RZ denotes the ratio of the Soret band absorbance to A_{280}), was purchased from Enzymology Inc., Columbus, OH, U.S.A., and was purified further by anion-exchange FPLC. *Arthromyces ramosus* (*Coprinus cinereus*) peroxidase was purchased from Suntory Ltd., Kyoto, Japan. Anionic tobacco peroxidase was purified from leaves of *Nicotiana sylvestris* plants, which overexpress this enzyme [16]. Lignin peroxidase H2 (LiP) and Mn-peroxidase H5 from *Phanerochaete chrysosporium* were kindly provided by Professor Ming Tien (Pennsylvania State University, State College, PA, U.S.A.), and cationic peanut peroxidase was generously given by Professor R. B. van Huystee (University of Western Ontario, London, Canada). Wild-type recombinant yeast cytochrome *c* peroxidase (CcP) expressed in *Escherichia coli* was obtained from Professor A. G. Cass (Imperial College, London, U.K.).

HPLC studies

Incubations were performed in 0.1 M potassium phosphate buffer, pH 7.0, at 22 °C unless stated otherwise. The reaction was initiated by the addition of enzyme; 200-μl aliquots were then taken and analysed by reverse-phase HPLC on a C_{18} Columbus column (300 Å, 5 μM, 150 mm × 4.6 mm; Phenomenex, Torrance, CA, U.S.A.) by using isocratic elution in a mixture of methanol/1 % acetic acid (2:3, v/v) at a flow rate of 1.0 ml/min. A Shimadzu LC-5A instrument was used in the experiments for measurement of A_{250}.

Oxygen uptake

Studies were performed with an oxygen electrode fitted with a plastic chamber (Rank Brothers, Cambridge, U.K.). Kinetic measurements were performed over a 0.1–6 mM range of IAA concentrations in 0.1 M potassium phosphate buffer, pH 7.0, at 22 °C.

Homology studies

Amino acid sequences of seven auxin-binding proteins were taken from GenBank (accession nos. are given in parentheses): *Arabidopsis thaliana* (X69901), *Capsicum annum* (Z48451), *Malus domestica* (U77952), maize (L08425, L08426) and *Nicotiana tabacum* (X70902, X70903). Alignments of amino acid sequences of haem-containing peroxidases were adapted from reviews published previously [17,18]. The sequence of anionic soya-bean peroxidase was taken from GenBank (AF007211). The search for structurally similar fragments was performed by using the MASAW program, version 2.0.0, with the BLAST algorithm [19]. Mapping of structurally similar fragments was performed by using the crystal structure of HRP [20] and RASMOL, version 2.6.

RESULTS AND DISCUSSION

IAA oxidation by oxygen catalysed by haem-containing peroxidases

IAA is known to be degraded in water solutions. However, in the absence of catalysts the process was very slow. In deionized water, IAA was relatively stable at 4 °C, and no degradation products were detected by HPLC for at least 24 h. Traces of IAA degradation products can be recorded after 5–6 h of incubation in distilled water at room temperature. To degrade IAA without the addition of plant peroxidases, one may use iron(II) chloride or haemin in a highly acidic medium [8]. In 1 % (v/v) acetic acid and in the presence of haemin (0.01 mg/ml), 1 mM IAA was degraded by 50 % in 1 h, yielding non-identified polymeric products. Iron(II) chloride (0.05 mg/ml) was also an efficient catalyst of IAA degradation under the above conditions. Methylene-3-oxindole is a major degradation end product when iron(II) chloride is used as a catalyst. The product spectrum produced during the course of degradation closely resembled that described for both horseradish and tobacco peroxidases [13], showing the presence of indole-3-methanol, indole-3-aldehyde, oxindole-3-carbinol and some minor non-identified products (results not shown).

Table 1 Oxygen consumption in the presence of 1 mM IAA in 0.1 M potassium phosphate buffer, pH 7.0, at 22 °C in the presence of 30 μM H_2O_2

The activity in the absence of hydrogen peroxide is shown in parentheses. ARP, fungal peroxidase from *Arth. ramosus*; MnP, manganese-dependent peroxidase from *Ph. chrysosporium*; PNP, cationic peanut peroxidase; SBP, soya-bean peroxidase; TOP, anionic tobacco peroxidase.

Enzyme	Concentration added (μM)	Initial rate (v; μM/min)	Duration of reaction (min)	O_2 consumed (%)
CcP	2.0	15 (0)	2	15 (0)
LiP	1.0	25 (0)	2	25 (0)
MnP	1.0	15 (0)	2	15 (0)
ARP	1.5	30 (0)	1	15 (0)
Microperoxidase	30	1.5 (0)	20	15 (0)
HRP	0.8	10 (10)	20 (20)	100 (100)
TOP	0.7	30 (14)	10 (13)	100 (100)
SBP	2.0	48 (2.0)	40 (100)	100 (100)
PNP	1.5	2.5 (2.5)	80 (80)	100 (100)

I - Active center of plant peroxidases (helix B, distal domain)

A.thaliana	91	**IHRHSC**	96
C.annum	74	**IHRHSC**	79
M.domestica	83	**IHRHSC**	87
Maize abp1	94	**IHRHSC**	99
Maize abp4	97	**IHRHSC**	102
N.tabacum T85	77	**IHRHSC**	82
N.tabacum T92	77	**IHRHSC**	82
HRPC	39	**LHFHDC**	44
PNP	39	**LHFHDC**	44
TOP	39	**LHFHDC**	44
SBP	39	**LHFHDC**	44
LiP	45	**LVFHDS**	50
MnP	44	**LTFHDA**	49
ARP(CiP)	53	**IVFHDA**	58
CcP	49	**LAWHTS**	54

II - the loop between helices D and D' (distal domain)

A.thaliana	84	**APGSETPIHRHSCEEVFVVL**	103
C.annum	67	**APGSSTPIHRHSCEEVFVVL**	86
M.domestica	75	**APGSGTPIHRHSCEEVFVVL**	94
Maize abp1	87	**SPGQRTPIHRHSCEEVFTVL**	106
Maize abp4	90	**GPGQRTPIHRHSCEEVFIVL**	109
N.tabacum T85	70	**APGSRTPIHRHSCEEIFVVL**	89
N.tabacum T92	70	**APGFRTPIHRHSCEEIFIVL**	89
HRPC	114	**GPSWRVPLGRRDSLQAFLDL**	133
PNP	114	**GASWNVLLGRRDSTTASLSS**	133
TOP	112	**GPSWQVLFGRKDSLTANRSG**	131
SBP	114	**GPHWKVPLGRRDSLTANRNL**	133
LiP	122	**APQMNFFTGRKPATQPA---**	138
MnP	121	**APRLEFMAGRPNTTIPA---**	137
ARP(CiP)	132	**SPRLEFLTGRSNSSQPS---**	148
CcP	121	**GPKIPWRCGRVDTPEDTTP-**	139

III - helix D (distal domain)

A.thaliana	95	**SCEEVFVVLKGSGTLYLAE**	113
C.annum	79	**SCEEVFVVLKGQGTLYLAP**	96
M.domestica	86	**SCEEVFVVLKGSGTLYLAP**	104
Maize abp1	98	**SCEEVFTVLKGKGTLLMGS**	116
Maize abp4	101	**SCEEVFIVLKGKGTLLLGS**	119
N.tabacum T85	81	**SCEEIFVVLKGQGILYLTP**	99
N.tabacum T92	81	**SCEEIFIVLKGQGTLYLTP**	99
HRPC	97	**SCADLLTIAAQQSVTLAGG**	114
PNP	97	**SCADILAVAARDSVVALGG**	114
TOP	95	**SCADILALASEIGVVLAKG**	112
SBP	97	**SCADILTLASQISSVLGGG**	114
LiP	104	TPGDFIAFAGAVALSNCPG	122
MnP	100	**SAADLVQFAGAVALSNCPG**	118
ARP(CiP)	112	**SFGDLIQFATAVGMSNCPG**	126
CcP	103	**SSGDLFSLGGVTAVQEMQG**	121

IV - helix A (distal domain)

A.thaliana	181	**PYYWDEQCIQESQKD**	196
C.annum	166	**PYYWDEECYQTTSSS**	181
M.domestica	174	**PYYWDEECLDVEPPP**	189
Maize abp1	185	**PFVWDEDCFEAAKDE**	200
Maize abp4	188	**PYFWDEDCLPAPKDE**	203
N.tabacum T85	179	**PYYWDEECYQTTSWK**	184
N.tabacum T92	179	**PYYWDEECYQTTSRK**	184
HRPC	4	**PTFYDNSCPNVSNIV**	18
PNP	4	**SNFYATKCPNALSTI**	18
TOP	4	**ATFYDTTCPNVTSIV**	18
SBP	4	**PSFYRDTCPRVHSIV**	18
LiP	7	KTVGDASCCAWFDVL	21
MnP	7	TRVTNAACCAFIPLA	21
ARP(CiP)	15	QSTSNSQCCVWFDVL	29
CcP	8	ASVEKGRSYEDFQKV	22

V - the beginning of helix C (distal domain)

A.thaliana	127	**ANSTIHIPIND**	138
C.annum	112	**PNSTFHVPVND**	123
M.domestica	120	**ANSTFHIPVND**	131
Maize abp1	131	**QNTTFSIPVND**	142
Maize abp4	134	**QNTTFSIPVND**	145
N.tabacum T85	125	**PNSTFHIPVND**	126
N.tabacum T92	125	**PNSTFHIPVND**	126
HRPC	71	**ANSARGFPVID**	82
PNP	71	**ANSIRGFEVID**	82
TOP	70	NVGAGGFD**IVD**	78
SBP	71	**NNSLRGLDVVN**	82
LiP	85	IGLDEVVAMQK	94
MnP	81	SGIDDSVNNLL	90
ARP(CiP)	93	GGLTDTVEALR	102
CcP	83	AGLQNGFKFLE	92

Figure 1 Amino acid sequences similar for both auxin-binding proteins and plant peroxidases

Auxin-binding proteins studied were those from *A. thaliana, Cap. annum, M. domestica*, maize and *N. tabacum*. ARP, fungal peroxidase from *Arth. ramosus*; HRPC, horseradish peroxidase isoenzyme C; MnP, manganese-dependent peroxidase from *P. chrysosporium*; PNP, cationic peanut peroxidase; SBP, soya-bean peroxidase; TOP, anionic tobacco peroxidase.

Fungal and yeast peroxidases were almost inactive at pH 3.5 compared with the horseradish enzyme, e.g. complete degradation of 100 μM IAA with 30 nM HRP was achieved in 30 min, whereas LiP and CcP, under similar conditions, showed only 2% conversion. The principal difference between the spectra of degradation products for the oxygenase reaction catalysed by plant and fungal peroxidases was the presence of indole-3-methanol for the former. This indicated the inability of fungal peroxidases to catalyse reactions consuming skatolyl hydroperoxide, which are shown in eqns. (9) and (10).

To avoid non-specific radical reactions of IAA degradation occurring in acid medium, we performed the experiment under neutral conditions, i.e. at pH 7.0. Since the rate of oxygen uptake was shown to correspond to that of IAA degradation [21], we followed the kinetics of oxygen consumption. In neutral media, both CcP and fungal peroxidases were essentially inactive towards IAA, as determined by HPLC and oxygen-uptake measurements (Table 1).

The addition of hydrogen peroxide should activate the common peroxidase pathway represented by eqns. (1)–(3), and IAA cation radicals formed should eventually yield skatolyl hydroperoxide via reactions (6)–(8). This could help to activate reactions (9) and (10), and thereby stimulate oxygen consumption, as has been shown previously for tobacco peroxidase [21]. As assessed by measurements of oxygen uptake in the presence of 1 mM IAA and in both the absence and presence of added hydrogen peroxide [catalysed by different haem-containing peroxidases (Table 1)], only plant peroxidases were able to catalyse the reaction without added hydrogen peroxide. However, in the case of anionic plant peroxidases (tobacco and soya bean), hydrogen peroxide served to remove an initial 'lag' period, indicating the differences in the initiation mechanism between anionic and cationic peroxidases. Oxygen consumption in the case of CcP and fungal peroxidases was observed only in the presence of hydrogen peroxide. Its duration was rather short, and the amount of oxygen consumed was approx. 1–2 mol. equivalents with respect to the amount of hydrogen peroxide added to the mixture. Thus the oxygen consumption in this case was due to the formation of IAA radicals via reactions (1)–(3), and their subsequent interaction with molecular oxygen via reaction (7). Skatolyl hydroperoxide, formed as shown in reaction (8), did not activate the oxygenase cycle. This suggests either an

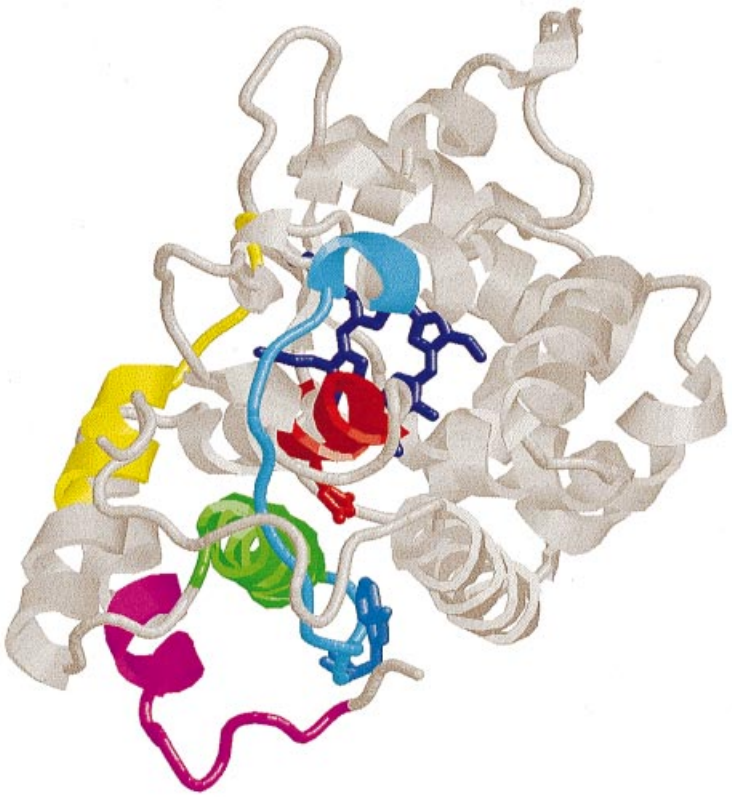

Figure 2 Fragments structurally similar to those found in auxin-binding proteins for HRP

Fragments structurally similar to those found in auxin-binding proteins are shown in the structure of HRP coloured red (amino acids 39–44), light blue (amino acids 114–133), green (amino acids 96–114), yellow (amino acids 71–82) and magenta (amino acids 4–18). Trp-117 is shown in dark blue and His-40 is in red. The peroxidase molecule is shown from the distal site.

inability of skatolyl hydroperoxide to act as an oxidative substrate, or an absence of a special binding site for IAA that would permit the reaction sequence (9) and (10). The data show unequivocally the absence of a hydrogen-peroxide-independent mode of activity towards IAA for microperoxidase and CcP and fungal peroxidases.

Structural similarity between auxin-binding proteins and classical plant peroxidases

The specificity of IAA oxidation for the enzyme structure, as described in the present study, enticed us to look for the structural basis for this phenomenon. Fortunately, both amino acid sequences and crystal structures are available for CcP [22], lignin [23] and Mn-peroxidases [24], *Cop. cinereus* [25] (*Arth. ramosus* [26]) peroxidase, peanut peroxidase [27] and HRP [20]. The peculiarities of the reaction mechanism, and the inability of prokaryotic and fungal peroxidases to catalyse the reaction of IAA oxidation, may be easily rationalized if there is a specific IAA-binding site in classical plant peroxidases.

The search for structural similarity between haem-containing peroxidases and auxin-binding proteins performed for the first time in the present study allowed us to identify five structurally similar fragments (Figure 1). It is evident that only classical plant peroxidases, i.e. tobacco, horseradish, soya-bean and peanut peroxidases, have fragments structurally similar to those of auxin-binding proteins. The corresponding fragments in CcP and fungal peroxidases show no similarity to auxin-binding proteins. Unfortunately, there is no information on the structures of the sequenced auxin-binding proteins, and thus the following discussion is on the basis of the structure of plant peroxidases.

Five structurally similar fragments presented in Figure 1 (I–V) form a subdomain (helices A, B, D–D′ and C) in the distal domain of plant peroxidase (Figure 2). The first structurally similar fragment (fragment I in Figure 1, and shown in red in Figure 2) corresponds to the sequence co-ordinating the haem from the distal site of the haem-binding pocket. This sequence belongs to helix B, and is highly conserved among all haem-containing peroxidases. However, only classical plant peroxidases contain two histidine residues in this fragment, namely His-40 and His-42. The sequences flanking this fragment in auxin-binding proteins are similar to the loop between helices D and D′ in plant peroxidases (residues 114–133 of fragment II in Figure 1, and shown in blue in Figure 2). This loop contains the tryptophan residue highly conserved among all classical plant peroxidases [17]. In the HRP crystal structure, Trp-117 is approx. 8–9 Å (0.8–0.9 nm) apart from nitrogen atoms of the porphyrin ring, and is in the zone of electron tunnelling. The fluorescence of the protein tryptophan residue has been recorded during the course of tyrosine peroxidation catalysed by HRP [28], and it has been suggested that it plays an important role in electron transfer through the peroxidase molecule.

Auxin-binding proteins also contain the fragment similar to helix D (residues 97–114 of fragment II in Figure 1; shown in green in Figure 2). Two other structurally similar fragments correspond to helix A (residues 4–18 of fragment IV, Figure 1; shown in magenta in Figure 2) and the beginning of helix C (residues 71–82 of fragment V in Figure 1; shown in yellow in

Figure 2) in the structure of HRP. Taken together, these five fragments form a subdomain including the catalytic centre and two residues highly conserved among classical plant peroxidases only, namely His-40 and Trp-117. The identified domain containing the above two residues could be directly involved in the oxidation of the physiological substrate of classical plant peroxidases, IAA.

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