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ORIGINAL ARTICLE

Mutagenesis, heterogeneous gene expression, and purification and amino acid substitution analyses of plant peroxidase, PrxA3a

Kyouji Yoshinaka · Shinya Kawai

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Abstract We introduced mutations into *prxA3a*, a peroxidase gene of hybrid aspen, *Populus kitakamiensis*, to substitute the amino acid residues at the surface of the protein, and analyzed substrate specificities. PrxA3a and mutated enzymes heterogeneously gene expressed in *Saccharomyces cerevisiae* were purified by Ni affinity chromatography, hydrolysis of sugar chain (Endoglycosidase H_f) and gel filtration. The substrate specificities were altered by substituted amino acid residues. PrxA3a F77Y A165W acquired the substrate specificity to *m*-chlorophenol. PrxA3a F77Y and PrxA3a F77YA165W could polymerize sinapyl alcohol. In addition, PrxA3a A165W, F77Y, and F77YA165W improved cytochrome *c* oxidizing activity. These substituted amino acid residues should function as a catalytic site outside of the heme pocket.

Keywords Peroxidase · Lignin · Sinapyl alcohol · Phytoremediation

Introduction

Peroxidases (EC 1.11.1.7) catalyze the oxidation of a variety of reductants by hydrogen peroxide [1, 2]. Plant peroxidases are involved in several physiological processes, e.g., the final step of monolignol oxidation in lignin biosynthesis [3, 4], the polymerization of extensin [5], the defense response to wounding [6, 7], the defense against pathogens [7, 8], and the metabolism of auxin [9, 10].

K. Yoshinaka (✉) · S. Kawai
United Graduate School of Agriculture,
Tokyo University of Agriculture and Technology,
3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
e-mail: yoshinaka_k@hotmail.com

Horseradish peroxidase C (HRP-C) is a typical plant peroxidase that has been used to study the mechanism of polymerization of lignin. But HRP-C is not present to any extent in tissues that are lignifying [11, 12]. HRP-C and HRP-A2 can oxidize coniferyl alcohol efficiently, but not sinapyl alcohol [13]. *Arabidopsis thaliana* peroxidase A2 (ATP-A2; 95% identity to HRP-A2) that was previously purified from the suspension culture of *A. thaliana* [14] could also oxidize coniferyl alcohol efficiently, but not sinapyl alcohol. Sinapyl alcohol could not bind to the substrate-binding site of ATP-A2, because its 5-methoxy group overlapped with Pro-139 of ATP-A2 [15]. This prediction was suggested to be generally applicable, because Pro-139 or proline residues in the corresponding positions are highly conserved in plant peroxidases (Fig. 1). For instance, 69 of 73 peroxidases in *A. thaliana* contain Pro-139 or Pro residue in the corresponding positions [13, 16]. Since HRP-C and ATP-A2 share high identities including Pro-139, HRP-C as a representative of plant peroxidases cannot catalyze sinapyl alcohol as a preferred substrate. PrxA3a, a peroxidase from hybrid aspen *Populus kitakamiensis*, has been concerned with lignin biosynthesis [1]; it also contains the proline residue corresponding to Pro-139 of ATP-A2 (Fig. 1) and oxidizes coniferyl alcohol efficiently, but not sinapyl alcohol.

On the other hand, cationic cell wall-bound peroxidase (CWPO-C) from poplar could catalyze the oxidation of sinapyl alcohol as preferred substrate [17–21]. Furthermore, CWPO-C could catalyze oxidation of synthetic lignin polymers and ferrocytochrome *c*, unlike other plant peroxidases [21]. But CWPO-C has not succeeded in the expression in yeast and plant cells (personal communication from Dr. Tsutsumi Y, Kyushu University). Fungal lignin peroxidase (LiP) could also catalyze the oxidation of lignin polymers and ferrocytochrome *c* [22]. CWPO-C and

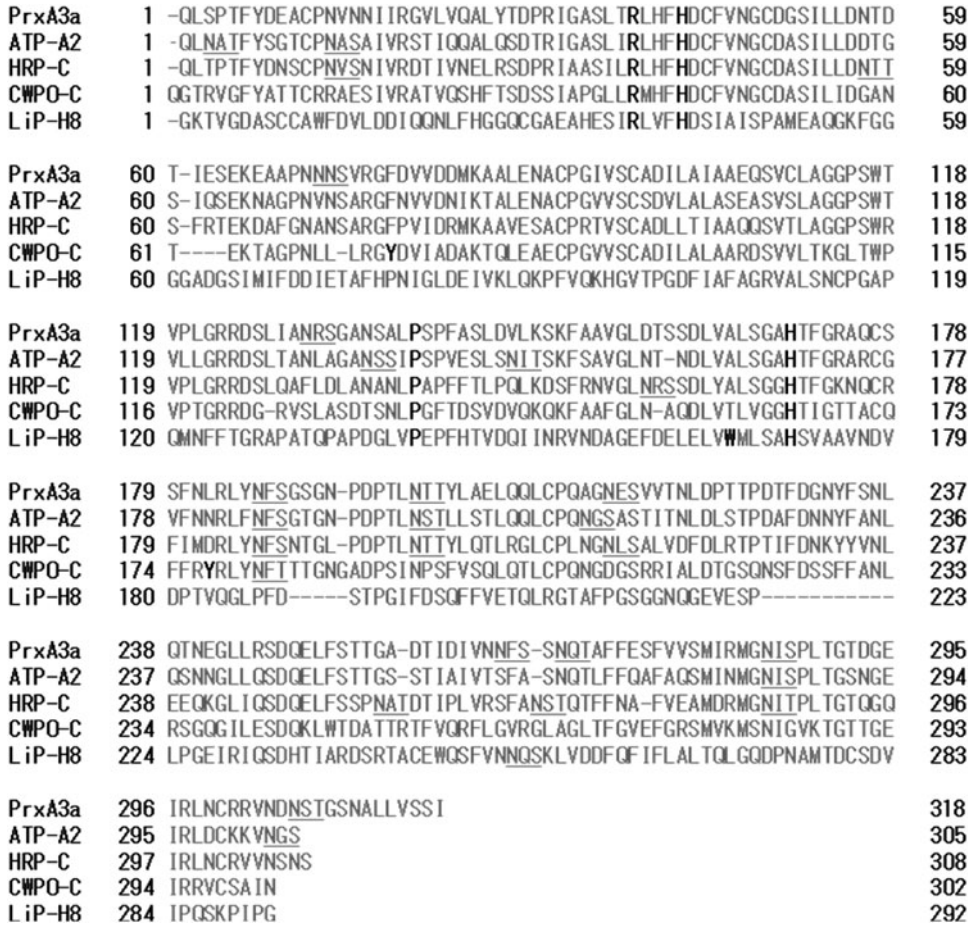

Fig. 1 Alignment of mature amino acid sequences for peroxidases PrxA3a, ATP-A2, HRP-C, CWPO-C and LiP-H8. Conserved amino acid residues in the hem pocket, proline residue corresponding to Pro-139 of ATP-A2, Trp-166 of LiP-H8, Tyr-74 of CWPO-C and Tyr-177 of CWPO-C are shown in *black*. *N*-glycosylation sites are *underlined*

LiP are suggested to be peroxidases with the additional active sites in the other positions other than the heme pockets. Therefore, these enzymes can also oxidize the substrate that cannot enter into the heme pocket according to a physical size etc. CWPO-C can catalyze sinapyl alcohol as a preferred substrate though Pro-135 corresponding to Pro-139 of ATP-A2 is preserved in CWPO-C. Therefore, amino acid residues located on the surface of the enzyme were forecasted as the catalytic site other than the heme pocket, and were investigated. As a result, it was clarified that Tyr-177 or Tyr-74 of CWPO-C was a catalytic site (Fig. 1) [23]. Tyr-177 and Tyr-74 located at the distance of <14 Å from the heme. Therefore, it is suggested that the long-range electron transfer with heme is possible [24–27]. LiP was also clarified that the Trp-171 residue located on the surface of LiP was a catalytic site [28–30].

Site-directed mutagenesis can introduce the mutation into a specific site. We tried to transplant the additional catalytic sites of CWPO-C and LiP to PrxA3a for substrate specificity modifications and for certifications of the surface amino acid residue hypothesis. The amino acid residues corresponding to the surface catalytic sites and the circumferential amino acid residues of catalytic sites in these enzymes were chosen for mutagenesis into *prxA3a* gene.

Materials and methods

Strains and plasmid

The host yeast strain used for production of PrxA3a and other mutated peroxidases was *Saccharomyces cerevisiae* BJ3505 (*pep4*::*HIS3 prb-Δ 1.6R HIS3*, *lys2-208*, *trp1-Δ101*, *ura3-52*, *gal2*, *can1*), a protease deficient strain, obtained from Sigma. The *Escherichia coli* strain used for construction and propagation of plasmids was XL10-gold (Tet^{r}Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte) [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r) Amy Cam^r].

Plasmid YEpFLAG-1 (Sigma) is a yeast vector for extracellular secretion of N-terminal FLAG fusion proteins in *S. cerevisiae*. Transcription is driven from *ADH2* inducible promoter and secretion occurs via the α-factor leader sequence. YEpFLAG-1 also contains the ampicillin

resistance gene, *bla*, for selection of transformed *E. coli* and *TRP1* for selection of yeast transformants.

Media and growth conditions

E. coli transformants were grown aerobically at 37°C in Luria–Bertani (LB) broth supplemented with ampicillin (100 μg/mL). *S. cerevisiae* transformants were selected at 30°C on SD solid medium containing 2% glucose, 0.67% yeast nitrogen base without amino acids, 0.2% casamino acids supplemented with 30 mg/mL lysine, 20 mg/mL uracil and 2% agar. Yeast transformant cells were precultured at 30°C to stationary phase in SD liquid medium without agar. A suitable volume of *S. cerevisiae* preculture medium was inoculated into YPHSM medium (1% yeast extract, 8% peptone, 1% glucose, 3% glycerol, 20 mM $CaCl_2$, 0.1% hemin, pH 7.0) at 30°C in a Sakaguchi flask and shaken with 135 rpm for 72 h.

Construction of plasmids

An anionic peroxidase gene, *prxA3a* was subcloned into pUC19 from a genomic library of *P. kitakaminensis* in λEMBL3 [1]. Introns were removed by mutagenesis performed with the Mutan®-K (TaKaRa) according to the manufacturer's instructions which are based on the method of Kunkel [31, 32] with intron removal primers (Fig. 2; Table 1). Site-directed mutagenesis of A165W was performed with mutated primers (Table 1). A fragment of about 1 kbp containing the complete coding region of *prxA3a* was amplified and the nucleotide sequence for the authentic signal sequence of PrxA3a was removed by PCR using KOD polymerase. The forward primer contained the sequence of an *Eco*RI restriction site and 6× His at the 5′ end (Table 1; Fig. 3). The reverse primer contained the sequence for a *Sal*I restriction site at the 5′ end (Table 1). The reaction mixture (50 μL) contained 0.2 mM each primer, 1 mM $MgCl_2$ and 0.6 U of KOD polymerase. The PCR reactions were carried out with incubation at 94°C for 2 min; 35 cycles of 94°C for 15 s, 62°C for 30 s, and 74°C for 30 s; and incubation at 72°C for 10 min. After amplification, the DNA fragment was digested with restriction enzyme *Eco*RI and *Sal*I (TOYOBO). And the digested fragment was inserted between the *Eco*RI and *Sal*I sites of plasmid YEpFLAG (Fig. 3) and introduced into *E. coli* XL10-Gold cells. Transformation was carried out by the $CaCl_2$ method [33]. Plasmid DNA was purified with the QIAprep® Spin Miniprep Kit (Qiagen). Other mutations were introduced by the site-directed mutagenesis using KOD polymerase as the following procedures. This mutagenesis method was an inverse PCR (iPCR)-based site-directed mutagenesis.

1. Inverse PCR of plasmid DNA, using a set of mutation primers (Table 1).
2. Plasmid DNA was digested with *Dpn*I. *Dpn*I can digest methylated DNA, such as plasmid DNA rescued from typical *E. coli* strains.
3. Self-ligation of PCR products is performed by a reaction with T4 polynucleotide kinase and ligase.
4. Transformation of *E. coli* using self-ligated PCR products.

Table 1 shows used primers. 28 plasmids for the mutated peroxidases were constructed.

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   1' atgatggtag ataaagcaat gcatccgtta gttgcatctc tttttattgt aatctggttt ggaggatcac
  71' ttccctatgc ctatgctCAG CTGAGTCCCA CTTTTTACGA TGAAGCATGC CCTAATGTGA ACAACATCAT
 141' TCGTGGAGTC CTTGTTCAGG CTTTGTACAC AGATCCCAGG ATTGGAGCCA GCCTCACTCG ACTTCATTTC
 211' CATGATTGTT TTGTTAATgt aaccatcttt ataactaatt atgttctttt tctaacattg ttagataatc
 281' aaacatgttg tttcttgaac ttcaagtgct aattatcagc ttggttcagG GTTGTGATGG ATCGATTCTT
 351' TTGGACAACA CTGATACTAT AGAGAGTGAG AAAGAAGCTG CTCCAAATAA TAATTCAGTA AGGGGTTTTG
 421' ATGTTGTTGA TGACATGAAG GCTGCACTAG AGAATGCTTG TCCTGGGATC GTCTCCTGTG CTGATATTCT
 491' TGCCATTGCA GCTGAACAGT CTGTTTGTTT Ggtatgttaa tgatttctct tgcctgtaac tattctcaaa
 561' ctttagtgaa tgttaaagaa agagtagaaa gcaaggagag ttttttttctt ttgtcaaatc atggactact
 631' aatatgctaa tttcgttgaa cctaaaattg aaaatgtgct aatccatgtg tctttgctgc agcttcttgt
 701' ttttggatac attgttgttt gcttcttaaa ttaatgtttg atcacagGCA GGAGGACCCT CATGGACAGT
 771' TCCTTTGGGA AGAAGAGACA GCTTAATAGC AAACAGAAGT GGTGCTAATT CTGCCCTTCC GTCTCCCTTC
 841' GCGAGCCTTG ATGTTCTCAA ATCCAAGTTT GCAGCCGTGG GCCTCGACAC CAGTAGTGAC CTGGTTGCCC
 911' TTTCAGgtat gtctttgtta gtattctgat ccatttatgt ctttttgcaa ggttttacat gcatgttttt
 981' cagGTGCTCA CACATTTGGA AGGGCTCAAT GTTCAAGTTT CAATCTTAGA TTGTATAATT TTAGTGGCAG
1051' TGGCAATCCT GATCCAACAC TGAACACAAC GTATCTAGCA GAACTTCAAC AATTATGTCC TCAAGCTGGA
1121' AATGAGAGCG TTGTAACAAA TCTTGATCCC ACTACACCTG ATACTTTCGA TGGCAACTAT TTTTCCAATC
1191' TTCAGACCAA TGAAGGCCTG CTTCGTAGTG ATCAAGAGTT GTTTTCTACT ACAGGTGCCG ATACCATTGA
1261' CATTGTCAAC AATTTTAGTA GTAATCAGAC TGCATTCTTT GAAAGCTTTG TGGTGTCCAT GATTAGAATG
1331' GGGAATATTA GCCCCTTAAC CGGGACAGAT GGAGAGATCA GATTGAATTG CAGAAGAGTC AACGACAACT
1401' CAACCGGATC AAATGCTCTC CTGGTTAGCT CAATTTAA
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Fig. 2 Nucleotide sequence of *prxA3a* and positions of intron elimination primers. Introns and the authentic signal sequence of PrxA3a are shown in *lower case*, and exons without the sequence for the authentic signal sequence are written in *upper case*. *Underlines* are shown selected sequences as primers

Table 1 Primers

	Forward primers	Reverse primers
For intron deletions		
Intron 1	ATGATTGTTTTGTTAATGGTTGTGATGGATC	
Intron 2	ACAGTCTGTTTGTTTGGCAGGAGGACCC	
Intron 3	GGTTGCCCTTTCAGGTGCTCACACATTTG	
*Eco*RI-6×His-*prxA3a*	GGAATTCCATCACCATCACCATCACCAGCTGAGTC	
*prxA3a-sal*I		GAAGGTCGACTTAAATTGAGCTAACCAG
For additional catalytic sites		
A165W	GCACCTGAAAGCCAAACCAGGTCAC	GTGACCTGGTTTGGCTTTCAGGTGC
L182Y	GAAGCTTGAACATTGAGCCCTTCC	AATTATAGATTGTATAATTTTAGTGGC
F76Y	GTACCCCCTTACTGAATTATTATTTGG	GATGTTGTTGATGACATGAAGGC
For alternation of circumference		
R245E	TGGAAAGTGATCAAGAGTTG	ACAAGCCTTCATTGGTCTG
F142T	ACTGCGAGCCTTGATGTT	GGGAGACGGAAGGGC
S178Q N181R	AAGCTCTGACATTGAGCCCTTCC	CAGATATCGATTGTATAATTTTA
S187T G188T S189T	ACCGGTAATCCTGATCCAACA	AGTAGTAAAATTATACAATC
N234D E235G	GGATCCGTTGTAACAAATCT	ATCTCCAGCTTGAGGACA
T159D	AGTGACCTGGTTTGGCTTTCAGGTG	AGAATCGTCGAGGCCCACGGC
D162E	TCTGAATTGGTTTGGCTTTCAGGTGCTCACAC	ACTGGTGTCGAGGCCCACGGCTGCA
I261E	GAAGTCAACAATTTTAGTAGTAATCAGA	ATCGATGGTATCGGCACCTGTAGTAGAAA
I261D	GACGTCAACAATTTTAGTAGTAATCAGA	ATCGATGGTATCGGCACCTGTAGTAGAAA
S275E	GTGTCCATGATTAGAATGGGGAATA	CACAAAATCTTCAAAGAATGCAGTCTGATTACTA
V278F	TTCTCCATGATCAGAATGGGGAATATTAGCCCCTT	CACAAAGCTTTCAAAGAATGCAGTC

Production of peroxidases in *S. cerevisiae* and purification

Recombinant plasmid (named YEpFLAG-6×His-*prxA3a*) was introduced into *S. cerevisiae* BJ3505 by the lithium acetate method [34]. Colonies of transformants showing a phenotype of the prototroph for tryptophan were selected on SD plates precultured transformed cells in SD liquid medium were transferred into YPHSM medium (1% yeast extract, 8% peptone, 1% glucose, 3% glycerol, 20 mM $CaCl_2$, 0.1% hemin, pH 7.0) at 30°C with shaking (135 rpm) in Sakaguchi flask for 72 h. Culture fluid was obtained by removing cells with centrifugation, and filtrated with a membrane filter (MILLEX-HV; Millipore).

Protein purification was done as following procedures:

1. Recombinant PrxA3a was purified from the resulting filtrate with a nickel affinity gel column (His-Select Cartridge; Sigma Aldrich Japan) according to the protocol supplied by the manufacturer.
2. Sugar chains were removed by Endoglycosidase H_f according to the protocol supplied by the manufacturer.
3. The second Ni affinity chromatography was performed.
4. Gel filtration was performed as the following procedures. Enzyme solution was buffer exchanged into MQ water using a Superdex 75 column. Bed dimensions were 10 mm diameter and 100 cm height. The flow rate was 0.33 mL/min and the sample volume was 200 μL. Peroxidase activity was detected using guaiacol oxidizing activity.

A single band on SDS-PAGE was obtained by purification in the order of the concentrate of the ultrafiltration, the His tag purification, the sugar chain elimination, the second His tag purification, and the gel filtration chromatography (Fig. 5). Protein concentrations were determined according to the method of Bradford using a Protein Assay kit (Bio-Rad).

Seven mutated enzymes, PrxA3a A165W, PrxA3a F77Y, PrxA3a F77Y A165W, PrxA3a L182Y, PrxA3a F77Y L182Y, PrxA3a L182Y R245E and PrxA3a L182Y R245E S178Q N181R, were chosen for further analyses because of their guaiacol oxidizing activities in cultured YPHSM media (it revealed good productivities and/or good performances of mutated enzymes) (data not shown), the side chain direction of Tyr-182 in homology modeling (PrxA3a L182Y R245E) and the results of TLC assays (PrxA3a F77Y A165W, PrxA3a L182Y R245E S178Q N181R). They were purified in the same way as recombinant PrxA3a.

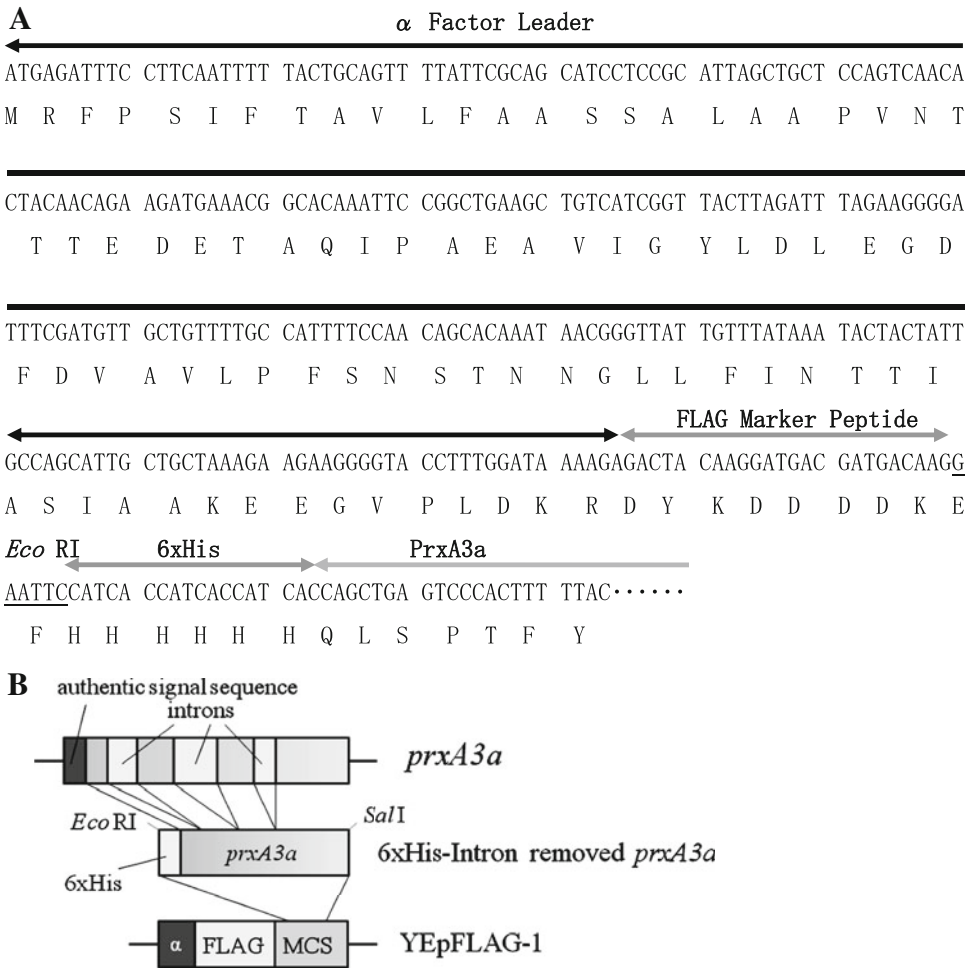

Fig. 3 Construction of plasmids. **a** Nucleotide and amino acid sequences for the amino terminal position of recombinant PrxA3a. It contains α factor leader sequence, FLAG marker peptide, *Eco*RI restriction site, 6× His and PrxA3a. **b** Structure of *Eco*RI-6×His-*prxa3a*-*Sal*I fragment

Enzyme activities

The oxidizing activity of PrxA3a was measured using guaiacol as substrate. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 0.1% (w/v) of guaiacol, 0.003% of hydrogen peroxide and an appropriate amount of enzyme. Oxidized guaiacol was measured by a spectrophotometer at a wavelength of 470 nm. One unit of enzyme activity was defined as the amount of enzyme that increased one unit absorbance at 470 nm (A_{470}) per minute in 1 mL reaction mixture at the room temperature. Optimum pH of enzymes was measured using the reaction mixtures with different pH.

TLC analyses

The substrate specificities of PrxA3a and mutated peroxidases were measured using *o*-chlorophenol, *m*-chlorophenol, *p*-chlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol, veratryl alcohol, *p*-*n*-nonylphenol, bisphenol A, 2-phenylphenol, 2-methoxy-4-methylphenol as substrates. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 0.1% (w/v) of each substrate, 0.003% of hydrogen peroxide and 1 U/mL of each enzyme. After the reaction for 2 h, reaction mixtures that added 100 μL of 1 N hydrochloric acid and 200 μL of ethyl acetate were stirred and centrifuged. An aliquot of the recovered organic fraction was spotted onto the TLC aluminum plate coated with silica gel containing a fluorescent indicator (Merck, #1.05554. 0009) and it was developed using development solvent (ethyl acetate:hexane:chloroform = 1:3:1). Spots were detected and photographed under UV irradiation.

Sinapyl alcohol oxidizing activities

The substrate specificities of PrxA3a and the mutated enzymes were measured using sinapyl alcohol as substrate. The reaction mixture consisted of 50 mM sodium acetate buffers (pH 7.0), 0.2 mM sinapyl alcohol, 0.003% of hydrogen peroxide and 0.268 μg/mL of each enzyme without sugar chains. UV spectrophotometer was used to measurement of temporal change in A_{285}.

For TLC analyses, the reaction mixture contained 0.4 mM sinapyl alcohol and 2.01 μg/mL of each enzyme without sugar chains and aliquots of the organic fractions

were developed using development solvent (ethyl acetate:acetone:chloroform = 2:5:2).

Cytochrome *c* oxidizing activities

The reaction mixture comprised 20 μM ferrocytochrome *c*, 0.268 μg/mL of PrxA3a or mutated PrxA3a, whose polysaccharides were removed, and 20 μM H_2O_2 in 50 mM Tris–HCl buffer (pH 7.5) containing 0.6 M NaCl. The reaction was initiated by adding the H_2O_2. The absorbance decrease at 560 nm, indicating oxidation of ferrocytochrome *c*, was monitored at room temperature [23].

Homology modeling of PrxA3a

Homology modeling of PrxA3a was performed using the Swiss Model server (http://swissmodel.expasy.org/). The ATP-A2 crystal structure (protein data bank entry 1pa2A) [35] was selected as the most appropriate template, since PrxA3a shares the highest amino acid sequence identity (69%) with ATP-A2 among plant peroxidases whose crystal structures have been reported.

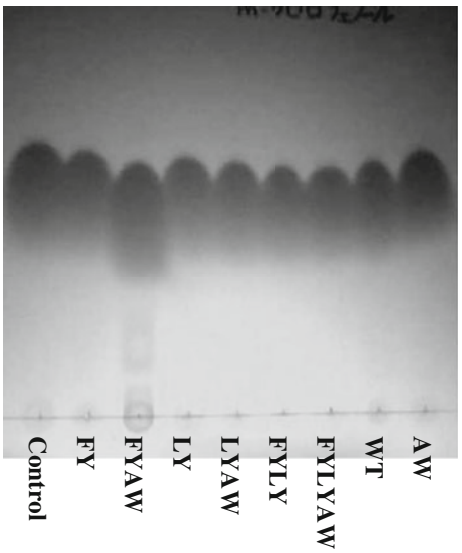

Fig. 4 TLC analysis of *m*-chlorophenol oxidizing activities. *WT* PrxA3a wild type, *AW* PrxA3a A165W, *FY* PrxA3a F77Y, *LY* PrxA3a L182Y, *FYAW* PrxA3a F77Y A165W, *LYAW* PrxA3a L182Y A165W, *FYLY* PrxA3a F77Y L182Y, *FYLYAW* PrxA3a F77Y L182Y A165W

Results and discussion

TLC analyses

Alterations of substrate specificities were observed. For example, PrxA3a F77Y A165W acquired *m*-chlorophenol oxidizing activity (Fig. 4). At TLC analysis for 2-phenyl phenol, PrxA3a L182Y R245E S178Q N181R and PrxA3a L182Y R245A S178Q N181R gave new faint spots (data not shown).

This TLC analysis was the qualitative research to determine whether mutated enzymes could oxidize each substrate or not. However, it was not suitable for the quantitative analysis on alternations of substrate specificities occurred by mutations to each chemical. Because guaiacol oxidizing activity of each enzyme was probably altered by the mutation, equal enzyme units for guaiacol did not mean equal amounts of respective mutated enzymes. For quantitative comparison of substrate specificities on TLC analysis or other analyses, it was necessary to use the equivalent amounts of enzymes. However, because the purification of enzyme was insufficient in the His tag purification, enzymes were crude. Then, each enzyme was purified furthermore.

Sugar chain elimination

There are putative nine *N*-glycosylation sites in PrxA3a (Fig. 1). Therefore, the band of PrxA3a did not appear at about 36 kDa on SDS-PAGE and native PAGE. Impurities were not removed by the Ni column, then enzymes were further purified using the ion-exchange chromatography and the gel filtration chromatography. The fractionation by the size of the protein was not performed in the gel filtration chromatography, and the different size protein passed the column at the same time (data not shown). The reason was assumed that the sugar chains of PrxA3a might be interactive with other proteins. Moreover, when the protein is crystallized to analyze the protein structure, it is necessary to be eliminated the sugar chains. Therefore, the sugar chains were eliminated during purification.

There are some methods of eliminating the sugar chains without damaging of the protein. For instance, there are the conversion of amino acid residues in *N*-glycosylation sites by site-directed mutagenesis, chemical treatment with trifluoromethansulfonic acid (TFMS), and hydrolysis with the enzyme.

By conversion of some *N*-glycosylation sites, peroxidase activities in cultured YPHSM media were decreased. It tended to greatly decrease protein secretion and/or peroxidase activity when mutations were introduced into three or more *N*-glycosylation sites including N264 and/or N268 (data not shown). Moreover, CWPO-C without sugar chain was not secreted from yeast (personal communication from Dr. Tsutsumi Y, Kyushu University). Therefore, sugar chains are probably important in the formation of the protein structure.

In the sugar chain removal from the enzyme, as a result of using Endoglycosidase H_f according to the protocol supplied by the manufacturer, we confirmed the sugar chain removed protein and its guaiacol oxidizing activity in a native PAGE (Fig. 5).

Purification and guaiacol oxidizing activities

PrxA3a and mutated enzymes were purified by Ni affinity chromatography, hydrolysis of sugar chains (by Endoglycosidase H_f), the second Ni affinity chromatography, and gel filtration (Table 2). The single bands on SDS-PAGE were obtained by as a result of the purification (Fig. 6). In further analyses, we utilized recombinant peroxidases whose sugar chains were eliminated. The specific activities were calculated. 0.067 μg of wild type PrxA3a without sugar chains was equivalent to 1 U of the peroxidase activity. Each 0.067 μg of purified peroxidases was added to the reaction mixture.

Clearly, the altered enzymes with L182Y mutation tended to decrease guaiacol oxidizing activities (Fig. 7).

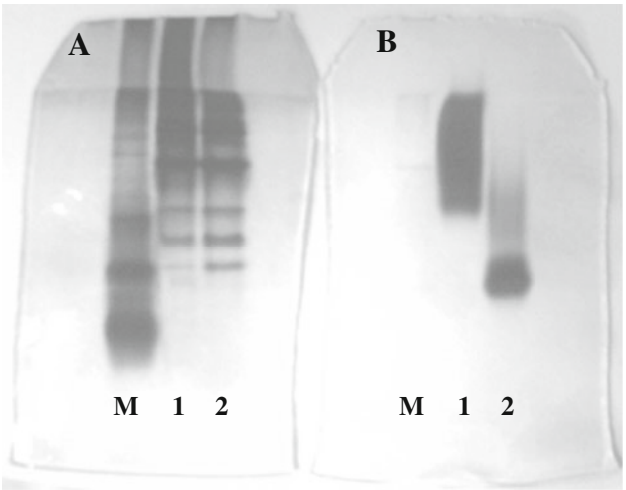

Fig. 5 Native PAGE of Endo H_f treated PrxA3a. **a** treated with silver stain, **b** treated with the guaiacol oxidizing activity stain. The size of the markers was inaccurate because it was undegeneration. *M* marker, *1* after Ni affinity chromatography, *2* after Endo H_f treatment

Comparison of specific activities of each enzyme for other phenols

Purified and sugar chain removed PrxA3a F77Y and Prx A3a F77Y A165W improved activities to *o*-chlorophenol

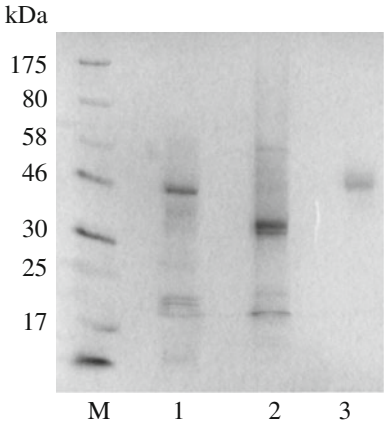

Fig. 6 SDS-PAGE of secreted proteins. *M* marker proteins, *1* PrxA3a secreted culture fluid, *2* after Ni affinity chromatography, *3* after Endo H_f treatment and gel filtration

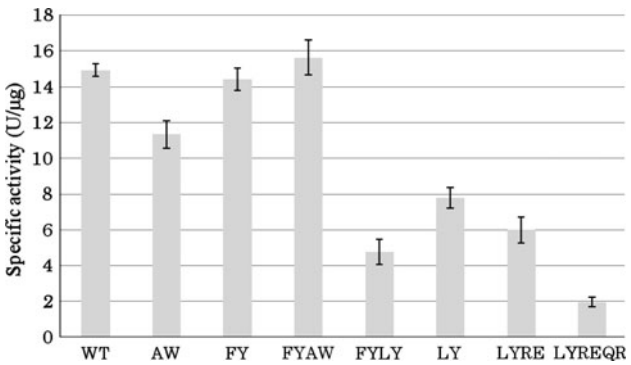

Fig. 7 Comparison of guaiacol oxidizing activities. *WT* PrxA3a wild type, *AW* PrxA3a A165W, *FY* PrxA3a F77Y, *LY* PrxA3a L182Y, *FYAW* PrxA3a F77Y A165W, *LYAW* PrxA3a L182Y A165W, *FYLY* PrxA3a F77Y L182Y, *FYLYAW* PrxA3a F77Y L182Y A165W, *LYRE* PrxA3a L182Y R245E, *LYREQR* PrxA3a L182Y R245E S178Q N181R. Their polysaccharide chains were eliminated. All assays were performed in triplicates. The *error bars* represent the 95% confidence interval of the mean of three independent experiments ($n = 3$)

Table 2 Purification table

Step	Volume (mL)	Total activity (U)	Total protein (μg)	Specific activity (U/μg)	Yield (%)	Purification factor
NM	400	14800	114000	0.129	100	–
NC	0.5	2400	1530	1.57	16.2	12.2
GF	6	989	81.4	14.4	6.70	112

NM nutrient medium after secretion, *NC* Ni affinity chromatography, *GF* after hydrolysis of sugar chains (by Endo H_f), second Ni affinity chromatography and gel filtration

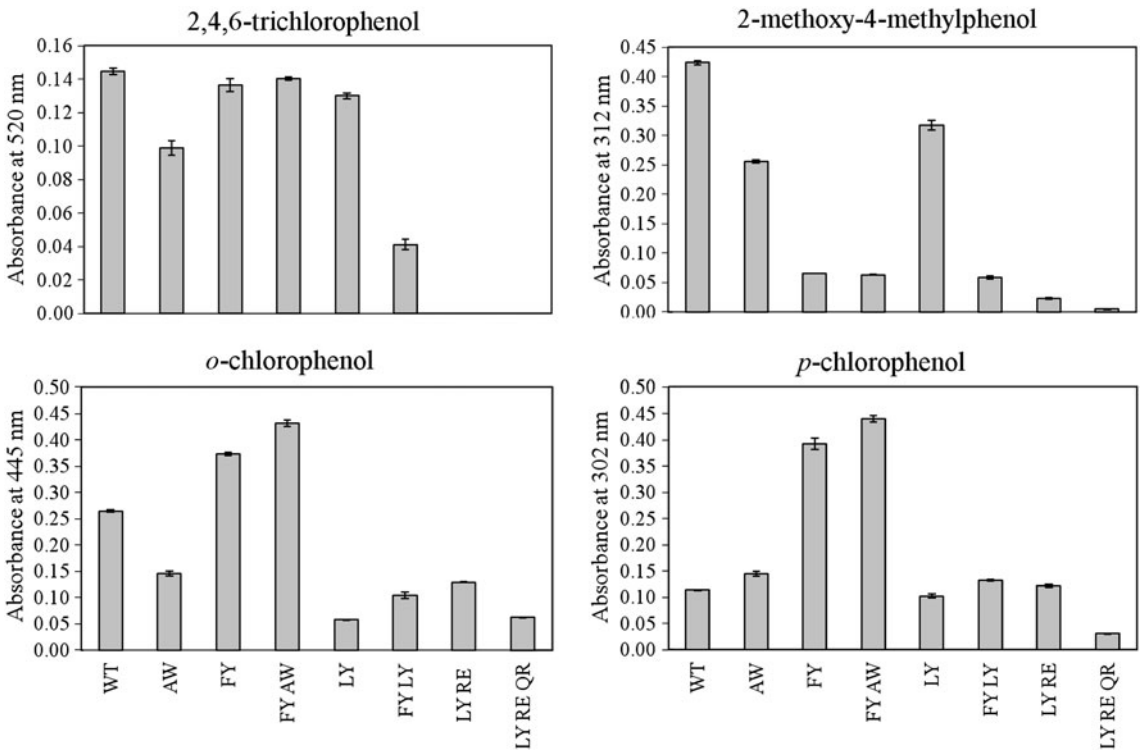

Fig. 8 Comparison of oxidizing activities for other phenols. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 6.0), 0.1% (w/v) of phenol, 0.003% of hydrogen peroxide and 0.067 μg/mL each enzyme (except 0.0067 μg/mL for 2-methoxy-4-methylphenol). Enzymes contained no sugar chains. Temporally changes of each absorbance for 3 min were measured. All assays were done in triplicates. A_{520} for 2,4,6-trichlorophenol, A_{312} for 2-methoxy-4-methylphenol, A_{445} for *o*-chlorophenol, A_{302} for *p*-chlorophenol. *WT* PrxA3a wild type, *AW* PrxA3a A165W, *FY* PrxA3a F77Y, *LY* PrxA3a L182Y, *FYAW* PrxA3a F77Y A165W, *LYAW* PrxA3a L182Y A165W, *FYLY* PrxA3a F77Y L182Y, *FYLYAW* PrxA3a F77Y L182Y A165W, *LYRE* PrxA3a L182Y R245E, *LYREQR* PrxA3a L182Y R245E S178Q N181R. The *error bars* represent the 95% confidence interval of the mean of three independent experiments ($n = 3$)

and *p*-chlorophenol, while these decreased the activity to 2-methoxy-4-methylphenol. The mutated enzymes with the mutation of L182Y intentionally decreased guaiacol oxidizing activity compared with PrxA3a. However, those enzymes also did not decrease activities to phenols other than guaiacol. For instance, PrxA3a F77Y L182Y slightly improved *p*-chlorophenol oxidizing activity than PrxA3a (Fig. 8).

Comparison of sinapyl alcohol oxidizing activities

PrxA3a F77Y without sugar chains improved sinapyl alcohol oxidizing activity (statistically significant). The enzymes with the mutation of L182Y decreased sinapyl alcohol oxidizing activities (Fig. 9).

Alterations of substrate specificities, especially *m*-chlorophenol oxidizing activity of PrxA3a F77Y A165W and improvement for sinapyl alcohol oxidizing activity of PrxA3a F77Y, were probably caused by new catalytic sites on the protein surface introduced by the mutations. Therefore, sinapyl alcohol polymerizing activity (whether

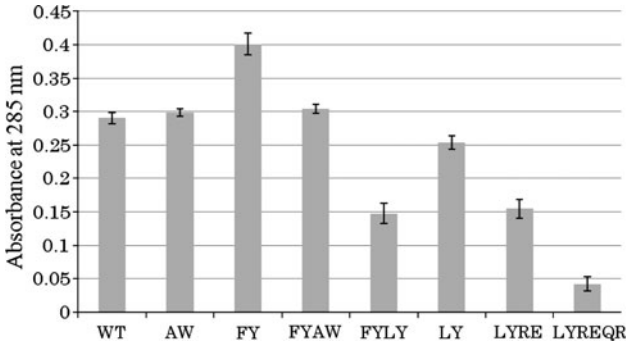

Fig. 9 Comparison of sinapyl alcohol oxidizing activities. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 6.0), 0.2 mM sinapyl alcohol, 0.003% of hydrogen peroxide and 0.268 μg/mL each enzyme without sugar chains. Temporally change in A_{285} for 10 min was measured. All assays were done in triplicates. *WT* PrxA3a wild type, *AW* PrxA3a A165W, *FY* PrxA3a F77Y, *LY* PrxA3a L182Y, *FYAW* PrxA3a F77Y A165W, *LYAW* PrxA3a L182Y A165W, *FYLY* PrxA3a F77Y L182Y, *FYLYAW* PrxA3a F77Y L182Y A165W, *LYRE* PrxA3a L182Y R245E, *LYREQR* PrxA3a L182Y R245E S178Q N181R. The *error bars* represent the 95% confidence interval of the mean of three independent experiments ($n = 3$)

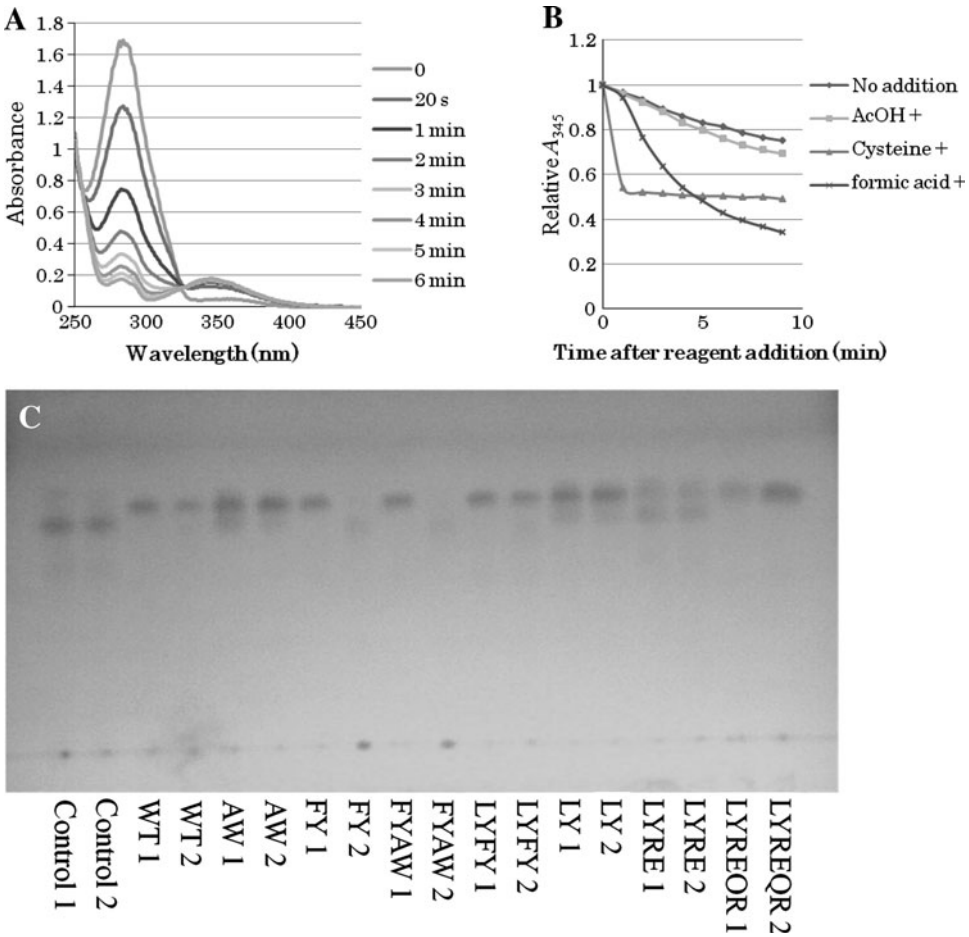

Fig. 10 Sinapyl alcohol polymerizing activities. **a** The peak of A_{285} decreased rapidly and the peak of A_{345} increased temporally when the PrxA3a excessive amount (2 μg/mL) was added. **b** The peak of A_{345} was derived from Syringyl-type quinone methide intermediate because it decreased rapidly by adding the acid, the oxidant, and the reducing agent. **c** *1* After 6 min reaction the A_{285} decrease was not measured. *2* Reaction mixtures incubated over night were developed on TLC. Spots from reaction mixtures of PrxA3a F77Y and PrxA3a F77Y A165W were different from others because those acquired sinapyl alcohol polymerizing activity. Sugar chains of enzymes were removed. *WT* PrxA3a wild type, *AW* PrxA3a A165W, *FY* PrxA3a F77Y, *LY* PrxA3a L182Y, *FYAW* PrxA3a F77Y A165W, *LYAW* PrxA3a L182Y A165W, *FYLY* PrxA3a F77Y L182Y, *FYLYAW* PrxA3a F77Y L182Y A165W, *LYRE* PrxA3a L182Y R245E, *LYREQR* PrxA3a L182Y R245E S178Q N181R

dimer or more polymerized) and cytochrome *c* (unable to enter in the heme pocket as substrate by its size) oxidizing activity were examined. On the TLC plate, clear spots appeared at three places (R_f 0.62, R_f 0.68, and the starting point) (Fig. 10c). The spot of R_f 0.62 that also appeared on the control lane was the spot of sinapyl alcohol monomer. In the result of reaction for 6 min, the monomer's absorption (A_{285}) decreased rapidly and A_{345} increased temporarily (Fig. 10a). The peak of A_{345} was derived from syringyl-type quinone methide intermediate because it decreased rapidly by adding the acid, the oxidant, and the reducing agent (It is known that the quinone methide compound makes unstably under the acid condition, and receives the nucleophilic addition with the nucleophilic reagent or the additional reaction of hydrogen by the reducing agent easily [36]) (Fig. 10b). A_{345} decreased gradually afterwards, and the reaction of making to the dimer progressed. Therefore, the spot of R_f 0.68 that appeared both after 6 min and after one night was suggested as the intermediate or dimer. As for PrxA3a F77Y and PrxA3a F77Y A165W, the spot of R_f 0.68 disappeared, and a new strong spot appeared on the starting point. It was suggested that the polymerization reaction had progressed further than dimer.

Cytochrome *c* oxidizing activities

A_{560} is one of absorption peaks of reduced cytochrome *c* (ferrocytochrome *c*), and it disappears in oxidized cytochrome *c* (ferricytochrome *c*). As for PrxA3a A165W, PrxA3a F77Y, and PrxA3a F77Y A165W, the absorbance has decreased intentionally compared with WT (Fig. 11). This showed that the rates of oxidation for reduced cytochrome *c* were improved. Especially, the oxidizing activity was greatly improved in PrxA3a F77Y A165W.

These results strongly supported the hypothesis that large molecular substrates unable to enter into the heme pocket of mutated PrxA3 are oxidized by Trp-165 and Tyr-77 located on the protein surface. In the case of LiP, site-directed mutations of Trp-171 were introduced into LiP-H8, and it was shown that the variant enzymes were unable to oxidize veratryl alcohol [37, 38]. ZePrx34.70 and ZePrx33.44 purified from *Zinnia elegans* could utilize sinapyl alcohol as their most comfortable substrate among

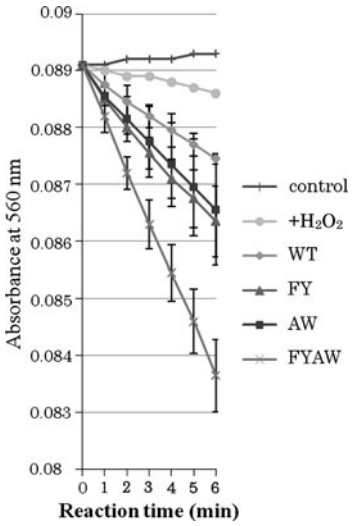

Fig. 11 Oxidation of ferrocytochrome *c*. Temporally decreases in A_{560}, indicating oxidation of ferrocytochrome *c*, were monitored. *WT* PrxA3a wild type, *AW* PrxA3a A165W, *FY* PrxA3a F77Y, *LY* PrxA3a L182Y, *FYAW* PrxA3a F77Y A165W, *LYAW* PrxA3a L182Y A165W, *FYLY* PrxA3a F77Y L182Y, *FYLYAW* PrxA3a F77Y L182Y A165W, *LYRE* PrxA3a L182Y R245E, *LYREQR* PrxA3a L182Y R245E S178Q N181R. Enzymes, whose polysaccharides were eliminated, were used. The *error bars* represent the 95% confidence interval of the mean of three independent experiments ($n = 3$)

three monolignols [39]. They also contain the corresponding tyrosine residue to Tyr-74 of CWPO-C but not the corresponding one to Tyr-177. These suggested that Trp-171 of LiP works as a catalytic site and that Tyr-74 of CWPO-C is necessary but enough to oxidize cytochrome *c* and to polymerize sinapyl alcohol.

On the other hand, enzymes with the mutation of L182Y decreased guaiacol oxidizing activity. However, it was suggested that not only the reduction of guaiacol oxidizing activity but also long-range electron transfer from the heme to the mutated site occurred as well as F77Y. In other words, the generation long-range electron transfer is the leakage of oxidizing power from the inside of the heme pocket to the outside. Tyr-182 was thought to be an occurrence of the electron transport easily than others because the position (10.3 Å) was nearer than ones of the other two surface amino acid residues (Trp-165 : 11.5 Å, Tyr-77 : 14 Å) from the heme. As a result, it is suggested that guaiacol oxidizing activity decreased because it is difficult for the substrate to be oxidized in the heme pocket that leaked the oxidizing power and because the protein structure and/or surrounding residues of Tyr-182 inhibit the entry of guaiacol into the heme pocket.

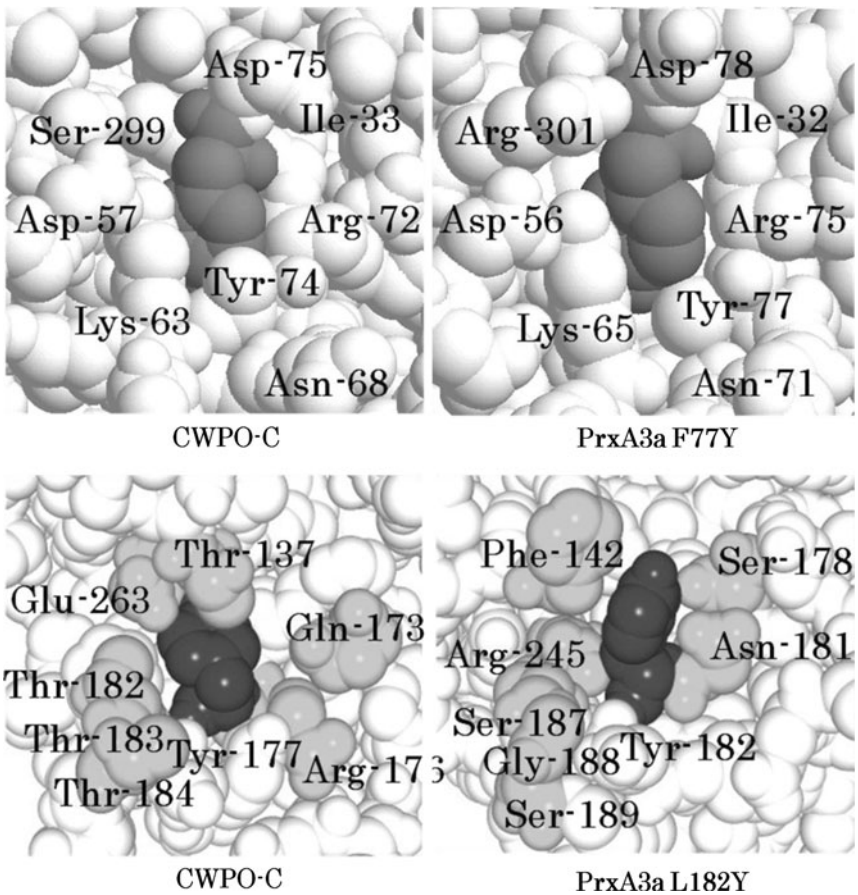

Fig. 12 Comparison of protein surfaces around the additional catalytic sites. Catalytic residues, Tyr-74, Tyr-77, Tyr-177 and Tyr-182, are shown in *dark*. The circumferential amino acid residues that were chosen for mutagenesis are shown in *gray*

The conservation of the amino acid residues around Tyr-177 of CWPO-C is low while the ones around Tyr-74 of CWPO-C are almost preserved when the amino acid sequence of CWPO-C is compared with that of PrxA3a (Fig. 12). For instance, the amino acid residue of PrxA3a corresponding to Glu-241 of CWPO-C is Arg, and even the side chain direction of Tyr-182 of PrxA3a was simulated to be different from one of Tyr-177 of CWPO-C according to this influence in homology modeling. Therefore, differences of peripheral amino acid residue of Tyr-182 perhaps prevented PrxA3a acquireing the ability of sinapyl alcohol polymerizing by the L182Y mutation. On the other hand, PrxA3a acquired the activity of sinapyl alcohol polymerization by the F77Y mutation with highly preserved peripheral amino acid residues. It suggests that the high activity of sinapyl alcohol polymerization of CWPO-C depends on Tyr-74 and peripheral amino acid residues. However, it does not deny the possibility that Tyr-177 functions as an additional catalytic site. Actually, HRP-C F179Y with the mutation corresponding to this position was confirmed that the Tyr residue was radicalized and HRP-C F179Y had the oxidizing activity of 3,3′,5,5′-tetramethylbenzidine [40]. As well as Tyr-77 of PrxA3a, we believe that further modifications of amino acid residues around Tyr-182 of PrxA3a are necessary to improve the mutated enzyme that can oxidize many substrates, instead of CWPO-C.

Conclusion

Site-directed mutations of Trp-165 and Tyr-77 were introduced into PrxA3a. These residues functioned as catalytic sites and mutated enzymes got sinapyl alcohol polymerizing activity and cytochrome *c* oxidizing activity. These results supported the hypothesis that the residues corresponding to these in CWPO-C and LiP act as additional catalytic sites.

A single band of PrxA3a on SDS-PAGE was obtained by purification. It is expected that it becomes the clue of the further modification of enzyme if the protein structure of PrxA3Aa can be clarified by the X-ray crystal structure analysis.

It is expected to contribute to breeding useful plant for phytoremediation and agricultural chemicals resistant crops if molecular breeding of the enzyme that improves the specificity of substrate to agricultural chemicals can be done.

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