JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 8883−8890 **8883**

Effect of Protein Structure on Laccase-Catalyzed Protein Oligomerization

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Laccase-catalyzed oligomerization of proteins was studied using *Trametes hirsuta* laccase (ThL) and coactosin as a model system. The reaction mechanism was elucidated using free amino acids and the tripeptide Gly-Leu-Tyr as substrates. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and high-performance liquid chromatography (HPLC) as well as oxygen consumption measurements and SDS-PAGE were used to study the reactions. Of the 15 selected amino acids, ThL was found to oxidize tryptophan (Trp), tyrosine (Tyr), and cysteine (Cys), of which the reactions with Tyr and Cys have been described earlier. ThL was able to link four full-length coactosins, whereas coactosin that was truncated from its C-terminus remained unpolymerized. Of the four tyrosine residues present in coactosin, only the tyrosine in the C-terminus was found to be reactive. Polymerization between tyrosine side-chains was unambiguously shown using different oligomers of Gly-Leu-Tyr as parent ions in MALDI-TOF/TOF MS fragment ion analyses.

KEYWORDS: Laccase; oligomerization; cross-linking; protein; tyrosine

INTRODUCTION

Laccases (EC 1.10.3.2) are multicopper and -domain blue proteins (MCBPs) (*1*) that utilize the distinctive redox potential of copper ions (*2*). They form a multicopper oxidase family (MCOs) together with the ascorbate oxidases (*3*) and ceruloplasmins (*4*, *5*). In nature, laccases are mainly found in fungi and plants (*6*−*10*), but some enzymes have been also isolated from bacteria and from insects (*11*−*15*). Fungal laccases are active in various environments. Some laccases are able to catalyze reactions, for example, at high temperatures (*16*) and at low pH (*17*). However, the optimal pH range for most laccases is 4.0 to ∼5.0 (*18*). Laccases also have a broad substrate specificity. They are capable of oxidizing various phenolic compounds, amines, thiols, and even some inorganic compounds such as iodine (*19*−*25*). Laccases oxidize their substrates by removal of one electron, which results in the formation of free radicals (*21*, *26*, *27*). The radicals can undergo further nonenzymatic reactions including disproportionation, polymerization, and hydration as well as fragmentation (*28*).

The ability of laccases to function in different conditions is important in terms of industrial applications. The application research has so far focused mainly on pulp and textile processes (*29*), whereas only limited food-related applications have been studied (*30*−*33*). Laccases have potential in, for example, food structure engineering as they can polymerize feruloylated carbohydrates (*34*, *35*) as well as tyrosine-containing proteins and peptides (*27*, *36*, *37*). In baking (*38*), laccases could improve bread volume (*39*) by cross-linking different sugars via ferulic acid side-chains (*35*, *40*, *41*). It has also been shown that laccases can cross-link whey proteins in the presence of phenolic acid (*42*). In meat applications, myosin heavy chain and troponin T were the most attractive substrates for laccases (*43*).

However, at present, laccase-catalyzed reactions with proteins at the residue level are poorly understood. Hitherto, the only known chemical bonds resulting from laccase-catalyzed oxidation of different amino acid side-chains have been the disulphide bonds formed between cysteines (*44*, *45*) and the isodityrosine bond formed between tyrosines (*27*).

In this work, we studied *Trametes hirsuta* laccase-catalyzed reactions with proteins to detect the production of oligomeric forms as well as to identify cross-links between separate protein molecules and between different amino acid side-chains in the protein. Because even small proteins have complex 3-D structures, 15 natural amino acids as well as the tripeptide Gly-Leu-Tyr were used as model substrates in addition to full-length and truncated actin filament-binding coactosin from *Mus musculus* (*46*). The 3-D structure of the full-length coactosin (**Figure 1**) consists of four α-helixes and seven β-sheets followed by a C-terminal region of 11 amino acids. Of its four tyrosine residues (14, 31, 45, and 137), only Tyr137 is located

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10.1021/jf062397h CCC: $33.50
Published on Web 10/24/2006

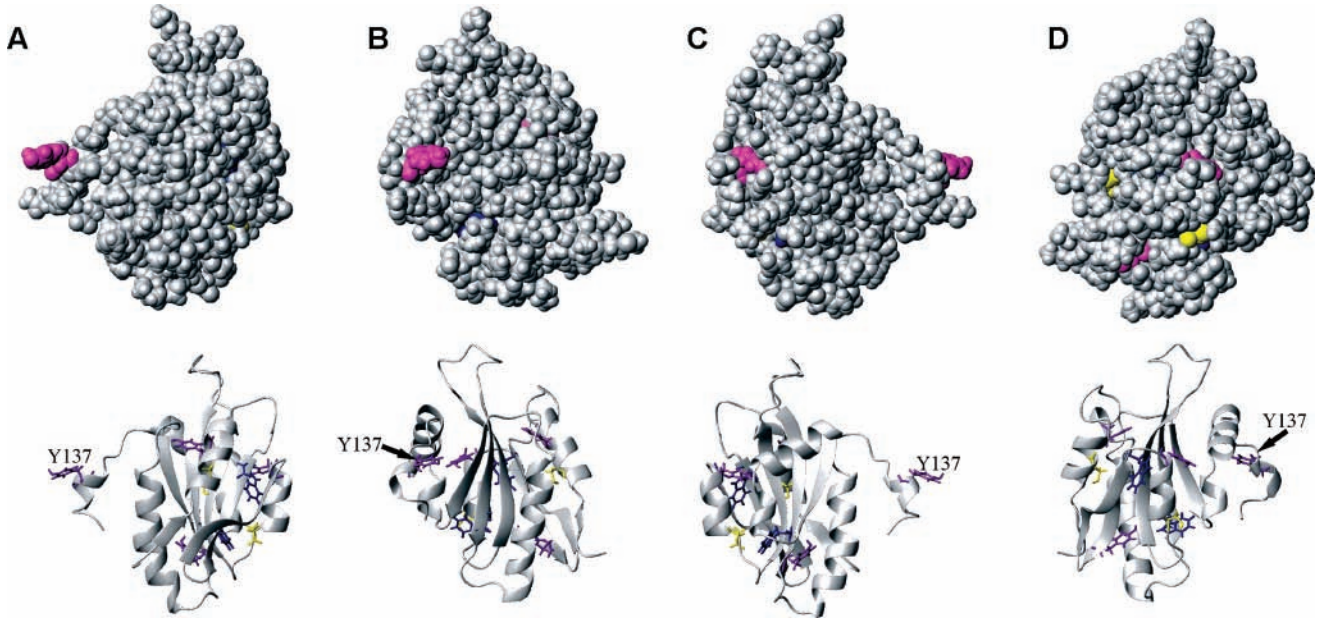


Figure 1. 3-D structures of full-length coactosin from different viewpoints (**A**–**D**). In the molecules, four tyrosines are shown in purple, two cysteines in yellow, and two tryptophans in blue. The figures were produced with the Protein Explorer program.

in the flexible tail and is thus expected to be easily accessible for oxidation and cross-linking. In the truncated coactosin, the C-terminal tail has been removed. Therefore, the full-length and truncated coactosins form an excellent model protein pair for enzymatic cross-linking studies.

MATERIALS AND METHODS

Enzyme. Laccase from the filamentous fungus *T. hirsuta* was purified and characterized at VTT (*47*), and its enzymatic activity was determined using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) as a substrate (*48*).

Substrates. Truncated coactosin (15 kDa, residues 1−131) was cloned to a pRAT5 plasmid (*49*) as described elsewhere for cloning of the full-length coactosin (16 kDa, residues 1−142) (*46*). Proteins were expressed in *Escherichia coli* BL21(DE3) cells in LB media. Both the full-length and the truncated proteins were purified as described previously (*46*). The yields of the purified full-length and truncated coactosins were 80 and 100 mg, respectively, from 1 L of cell culture. Amino acids used as ThL substrates in oxygen consumption measurements were of a different origin. Phenylalanine, tyrosine, and cysteine were from Merck (Darmstadt, Germany); proline, serine, threonine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, histidine, leucine, and lysine were from Fluka (Buchs, Switzerland); and tryptophan was from Sigma-Aldrich (St. Louis, MO). The tripeptide Gly-Leu-Tyr was purchased from Sigma-Aldrich (St. Louis, MO).

Enzyme Treatment. Depending on the method, enzymatic reactions were carried out at pH 4.5 or 6.0 in 25 or 10 mM succinic acid. To speed up the reactions to a reasonable level as well as to increase the amounts of the reaction products to detectable levels, rather high enzyme dosages (70−4000 nkat/μmol of substrate) were used. However, before the experiments, it was ensured that the reactions also occurred in catalytic enzyme concentrations (i.e., when the enzyme−substrate ratio was c.a. 1:1000). The pH optimum of ThL is 4.5, and hence, the catalytic activities of the enzyme on the free amino acids and on the tripeptide Gly-Leu-Tyr were determined at this pH, although the coactosins are not fully stable at pH 4.5. However, when evaluating the relevance of the 3-D structure of the full-length coactosin for the enzyme activity, the experiments were performed at pH 6.0. The concentrations of the model substrates varied between 0.06 and 1.5 mM with incubation times of 1−24 h. To prevent aggregation of the protein, 1 mM dithiothreitol (DTT) was added to the reaction mixture. To perform the gel-filtration experiments with the full-length coactosin, 4 mg of pure protein was treated with the enzyme in the same reaction conditions as used in the SDS-PAGE experiments. All enzyme reactions were performed at room temperature.

Oxygen Consumption Measurements. The ThL-catalyzed oxidation of selected amino acids as well as the reactivity of the enzyme on the full-length and truncated coactosin were analyzed by monitoring the consumption of dissolved oxygen during the ThL reaction. After initiation of the reaction by enzyme addition, oxygen consumption was monitored for 3 h using an oxygen electrode (FIBOX 3 fiberoptic oxygen meter, PreSens, Germany). The measurements were carried out under constant mixing in completely filled 1.84 mL sealed flasks to avoid entry of oxygen into the reaction mixture during the measurements.

SDS-PAGE. Proteins were separated by SDS-PAGE in 12% polyacrylamide (w/v) gels according to Laemmli (*50*) and subsequently stained with Blue Stain Reagent (Pierce Biotechnology Inc., Rockford, IL). Molecular weight standards (6.5−66 kDa, prestained standard, broad range, Bio-Rad, Hercules, CA and 14.4−94 kDa, low molecular weight marker, Amersham Biosciences, Uppsala, Sweden) were used as molecular weight markers. The gel images were analyzed using a BioRad GelDoc 2000 gel-documentation system equipped with a Quantity One program (Bio-Rad Laboratories, Hercules, CA).

Gel-Filtration. Reaction products resulting from ThL-catalyzed reactions on full-length coactosin were subjected to gel-filtration in a Superdex 75 16/60 gel-filtration column. Chromatography was performed in 10 mM bis-Tris, pH 6.0, 50 mM NaCl, 1 mM DTT, and 0.1% NaN_3 using an ÄKTA Purifier liquid chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). Fractions of 1 mL were collected and subsequently analyzed by SDS-PAGE.

Trypsin Digestion and Protein and Peptide Separation by RP-HPLC. For trypsin digestion, the fractions from gel-filtration corresponding to monomeric and dimeric forms of full-length coactosin were subjected to reversed-phase chromatography in a 2 mm × 20 mm TSK-TMS250 column (TosoHaas, Tokyo, Japan). Chromatography was performed by a linear gradient of acetonitrile (3−100% in 60 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 200 μL/min and by monitoring at 214 nm. The peaks corresponding to the monomeric and dimeric forms on the basis of SDS-PAGE were alkylated and digested with trypsin, and the tryptic peptides were separated by reversed-phase chromatography on a 1 mm × 150 mm C4 (214TP5115, Grace Vydac, Hesperia, CA) column (*51*).

Mass Spectrometry. MALDI-TOF/(TOF) MS analyses were performed on an Ultraflex TOF/TOF MS (Bruker Daltonik GmbH, Bremen, Germany) instrument equipped with a nitrogen laser operating at 337 nm. The mass spectra were acquired in positive ion reflector

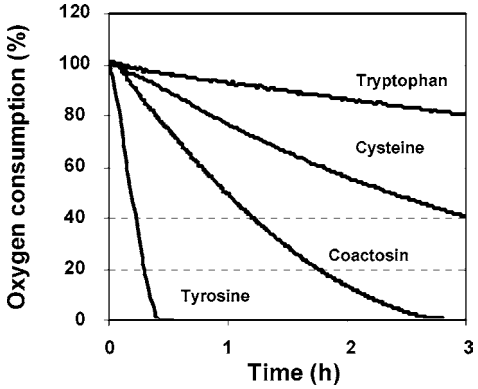


Figure 2. Oxygen consumption vs time in ThL-catalyzed reactions with tyrosine, cysteine, and tryptophan as well as with full-length and truncated coactosin. Treatment conditions for the amino acids were as follows: volume 1.84 mL, ThL dosage 70 nkat/μmol, buffer 25 mM succinic acid, pH 4.5, substrate concentration 1.5 mM. Treatment conditions for the full-length and truncated coactosin were as follows: ThL dosage 140 nkat/μmol, buffer 10 mM succinic acid, pH 4.5, substrate concentration 1 mM.

mode using α-cyano-4-hydroxycinnamic acid (Aldrich, Steinheim, Germany) as the matrix and external calibration with a peptide calibration standard (P/N 206195, Bruker Daltonik, Bremen, Germany). For MALDI-TOF/TOF fragment ion analysis of selected parent ions, the instrument was operated in the LID-LIFT mode.

RESULTS

ThL-Catalyzed Oxidation of Selected Amino Acids and a Model Peptide. The suitability of 15 naturally occurring amino acids as substrates for ThL was tested by the oxygen consumption method. Serine and threonine with a hydroxyl group in the side-chain were selected from the nonaromatic amino acids. All acidic, basic, and aromatic amino acids as well as imino acids were tested. Cysteine from sulfur-containing amino acids as well as leucine from the aliphatic amino acids were also tested. On the basis of the oxygen consumption measurements, ThL could oxidize tryptophan, cysteine, and tyrosine (**Figure 2**), of which the reactivities with cysteine (*44*, *45*) and tyrosine (*27*) have been reported earlier. Oxygen consumption rates were estimated from the linear slopes of the curves to be 0.05×10^{-12}, 0.16×10^{-12}, and 1.4×10^{-12} mol s^{-1} $nkat^{-1}$ (*27*), respectively. The error of the measurements was approximately ±10%. The ability of ThL to oxidize cysteine and tryptophan as compared to tyrosine was weak, and hence, ThL reactivity toward these model compounds was not further studied. The oxygen consumption rate for the tripeptide Gly-Leu-Tyr (3.8×10^{-12} mol s^{-1} $nkat^{-1}$) has been published recently (*27*).

The oligomerization of the tripeptide Gly-Leu-Tyr by ThL and the covalent linkages formed between tyrosine residues in the catalysis were studied by MALDI-TOF MS. The mass spectrum measured directly from the reaction mixture after 30 min reaction time shows clear oligomerization of the peptide, and homopolymers up to 11 units were detected from the solution (**Figure 3A**). The covalent bonds between the monomers were formed by elimination of two hydrogen atoms as shown previously (*27*). Hence, the calculated molecular masses for the oligomers are given by $[n\mathrm{MW} - (n - 1)2\mathrm{H} + \mathrm{H}]^+$, where n is the number of monomers and MW is the molecular weight of the substrate. The observed monoisotopic masses as well as the corresponding predicted masses of the oligomers are listed in **Table 1**. The differences are within the mass accuracy of the used instrument.

The nature of the chemical bonds in the formed Gly-Leu-Tyr oligomers was further studied by MALDI-TOF/TOF MS

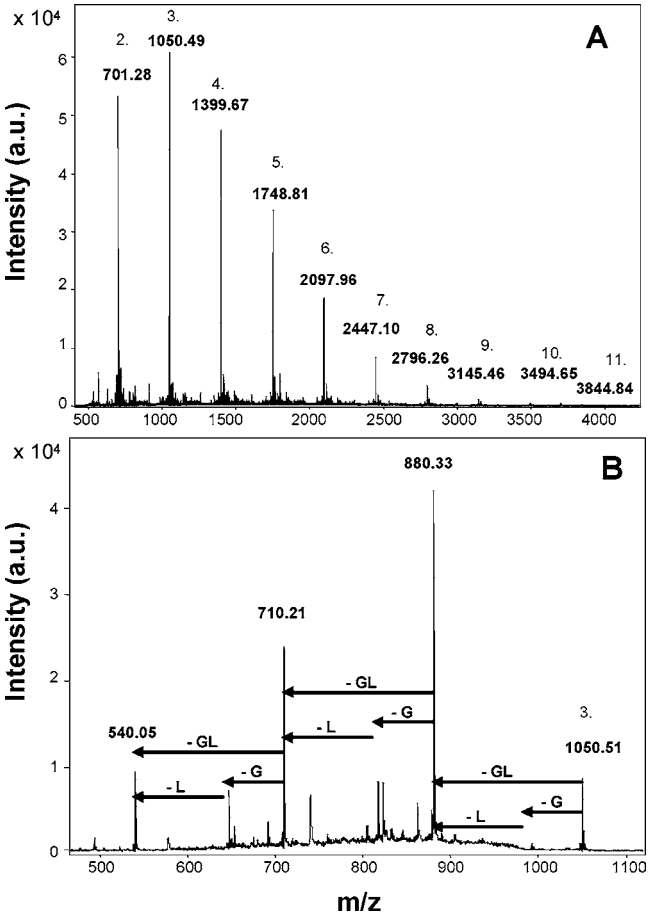


Figure 3. Mass spectra measured from ThL-treated Gly-Leu-Tyr tripeptide. Treatment conditions: volume 0.1 mL, ThL dosage 70 nkat/μmol, buffer 25 mM succinic acid, pH 4.5, substrate concentration 1.5 mM, reaction time 30 min. In the MALDI-TOF mass spectrum (**A**), the Arabic numbers above the monoisotopic protonated masses show the number of Gly-Leu-Tyr monomers in the oligomers, resulting in the obtained masses. In the MALDI-TOF/TOF MS experiment (**B**), the trimeric molecule with a determined monoisotopic protonated mass of 1050.49 Da (corresponding to three covalently bound Gly-Leu-Tyr monomers) was used as the precursor ion and analyzed in the LID-LIFT mode without collision gas. The resulting fragment ions correspond to a sequential loss of Gly and Leu fragment ions resulting in a tyrosine fragment trimer with a protonated mass of 540.05 Da.

Table 1. Monoisotopic Protonated Masses of Oligomers Resulting from Incubation of Gly-Leu-Tyr Tripeptide with ThL[a]

oligomer	observed mass (*m/z*)	predicted mass (*m/z*)	difference (*m/z*)
$[(GLY)_2H]^+$	701.28	701.35	–0.07
$[(GLY)_3H]^+$	1050.49	1050.51	–0.02
$[(GLY)_4H]^+$	1399.67	1399.68	–0.01
$[(GLY)_5H]^+$	1748.81	1748.84	–0.03
$[(GLY)_6H]^+$	2097.96	2098.00	–0.07
$[(GLY)_7H]^+$	2447.10	2247.17	–0.06
$[(GLY)_8H]^+$	2796.26	2796.33	–0.07
$[(GLY)_9H]^+$	3145.46	3145.49	–0.03
$[(GLY)_{10}H]^+$	3494.65	3494.66	–0.01
$[(GLY)_{11}H]^+$	3844.84	3843.48	–1.36

[a] Corresponding MALDI-TOF mass spectrum is shown in **Figure 3A**. The predicted masses of the oligomers are shown for comparison as well as the differences between observed and predicted masses.

as shown in the example for the Gly-Leu-Tyr trimer. When the Gly-Leu-Tyr trimer (m/z = 1050.49 Da) was fragmented by laser-induced dissociation (LID) and analyzed in the LIFT mode,

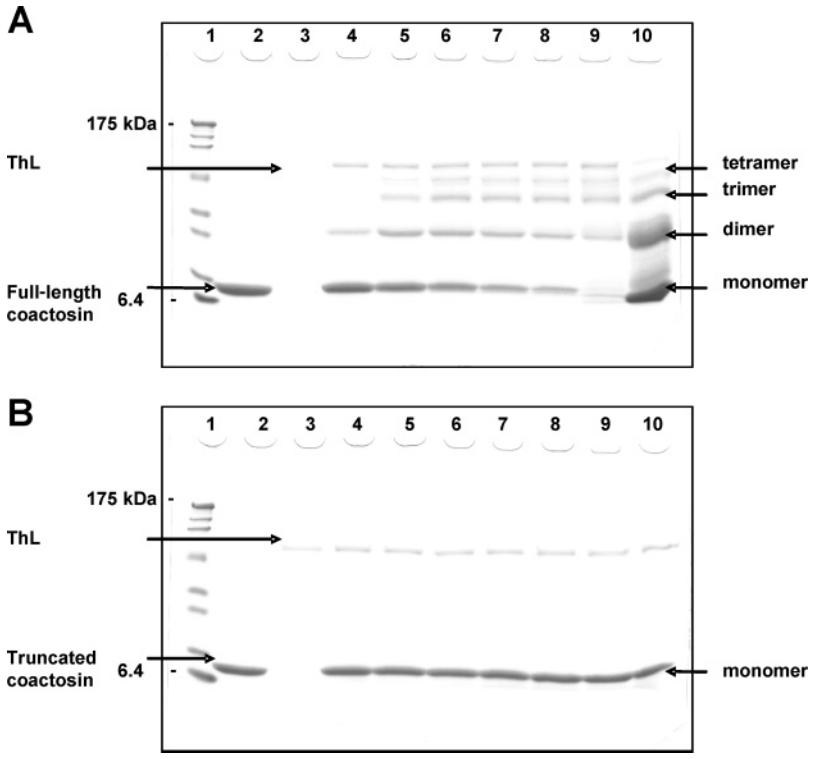


Figure 4. SDS-PAGE analyses of ThL-treated full-length and truncated coactosin. (**A**) In lane 1: molecular weight marker (6.5, 16.5, 25, 32.5, 47.5, 62, 83, and 175 kDa); lane 2: full-length coactosin; lane 3: ThL; lanes 4–9: time points (2, 3, 4, 5, 6, and 24 h) from the ThL–full-length coactosin reaction mixture; and lane 10: sample from the oxygen consumption measurement (**Figure 2**) of full-length coactosin. (**B**) In lane 1: the same molecular weight marker as in panel A; lane 2: truncated coactosin; lane 3: ThL; and lanes 4–10: time points (1, 2, 3, 4, 5, 6, and 24 h) from the ThL–truncated coactosin reaction mixture. Treatment conditions: volume 0.02 mL, buffer 10 mM succinic acid, pH 6.0 containing 1 mM DTT, substrate concentration 0.06 mM for full-length or truncated coactosin, ThL dosage 4000 nkat/μmol.

a sequential loss of Gly and Leu fragment ions was detected (**Figure 3B**), with the final product (m/z = 540.05 Da) corresponding to a covalently bound tyrosine fragment trimer.

ThL-Catalyzed Oligomerization of Model Proteins. According to the oxygen consumption measurement, ThL could also oxidize a small protein, the full-length coactosin, which contains four tyrosine, two tryptophan, and two cysteine residues in its polypeptide chain. The oxygen consumption rate was 0.4 $\times$ 10^{-12} mol s^{-1} $nkat^{-1}$. By contrast, truncated coactosin could not be oxidized by ThL (data not shown). As can be seen from **Figure 2**, oxygen consumption curves of the model substrates decayed in the order tyrosine > coactosin > cysteine > tryptophan. Oxygen consumption rate was the highest for tyrosine, but surprisingly the rate for the full-length coactosin protein was only 3.5 times slower than that for tyrosine.

The molecular weights of the reaction products of ThL-oxidized coactosin were determined by SDS-PAGE. The degrees of polymerization of the full-length and truncated coactosins formed in ThL-catalyzed reactions are shown in **Figure 4**. In the case of full-length coactosin (**Figure 4A**), molecular weights corresponding to oligomers up to tetramers were formed after a reaction time of 2 h (**Figure 4**, lane 4). After a 24 h reaction time, nearly all the coactosin monomers had been polymerized (**Figure 4**, lane 9). A sample from the end point of the oxygen consumption measurement (**Figure 4A**, lane 10) is shown for comparison. On the basis of the intensities of the bands after 24 h reaction time (**Figure 4**, lane 9), only approximately 10% of the original intensity of the full-length, unpolymerized coactosin could be detected. In the case of truncated coactosin, without the flexible C-terminal tail (**Figure 4B**), no oligomerization products were observed. Only the bands corresponding to the truncated coactosin monomer and ThL were visible on the gel. These results suggest that oligomerization of the full-length coactosin occurred via the flexible C-terminal tail containing the accessible tyrosine residue (Tyr137). To further support these findings, equal amounts of the full-length and truncated coactosin were mixed together and treated with ThL for 1, 6, and 24 h. As can be seen from **Figure 5**, lanes 5−7, the band of the full-length coactosin decreased as a function of the reaction time, and after 24 h, the band had almost disappeared, whereas the band of truncated coactosin still had its original intensity, indicating that the truncated coactosin remained unpolymerized in ThL treatment. By contrast, the full-length coactosin polymerized up to dimers, trimers, and tetra-

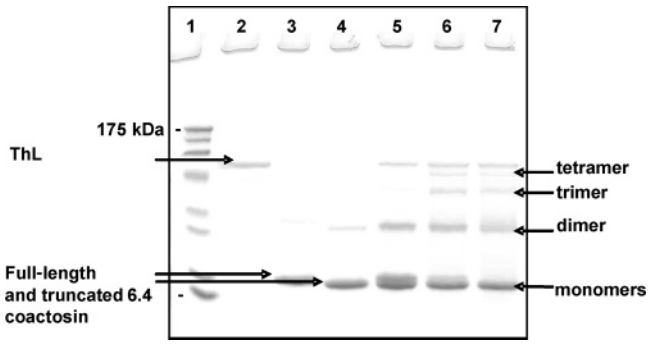


Figure 5. SDS-PAGE analysis of a ThL-treated mixture of full-length and truncated coactosin. Lane 1: molecular weight marker (6.5, 16.5, 25, 32.5, 47.5, 62, 83, and 175 kDa); lane 2: ThL; lane 3: full-length coactosin; lane 4: truncated coactosin; and lanes 5–7: time points (1, 6, and 24 h) from the mixture of full-length and truncated coactosin treated with ThL. Treatment conditions: volume 0.02 mL, buffer 10 mM succinic acid, pH 6.0, substrate concentration 0.04 mM for full-length and truncated coactosin, ThL dosage 400 nkat/μmol.

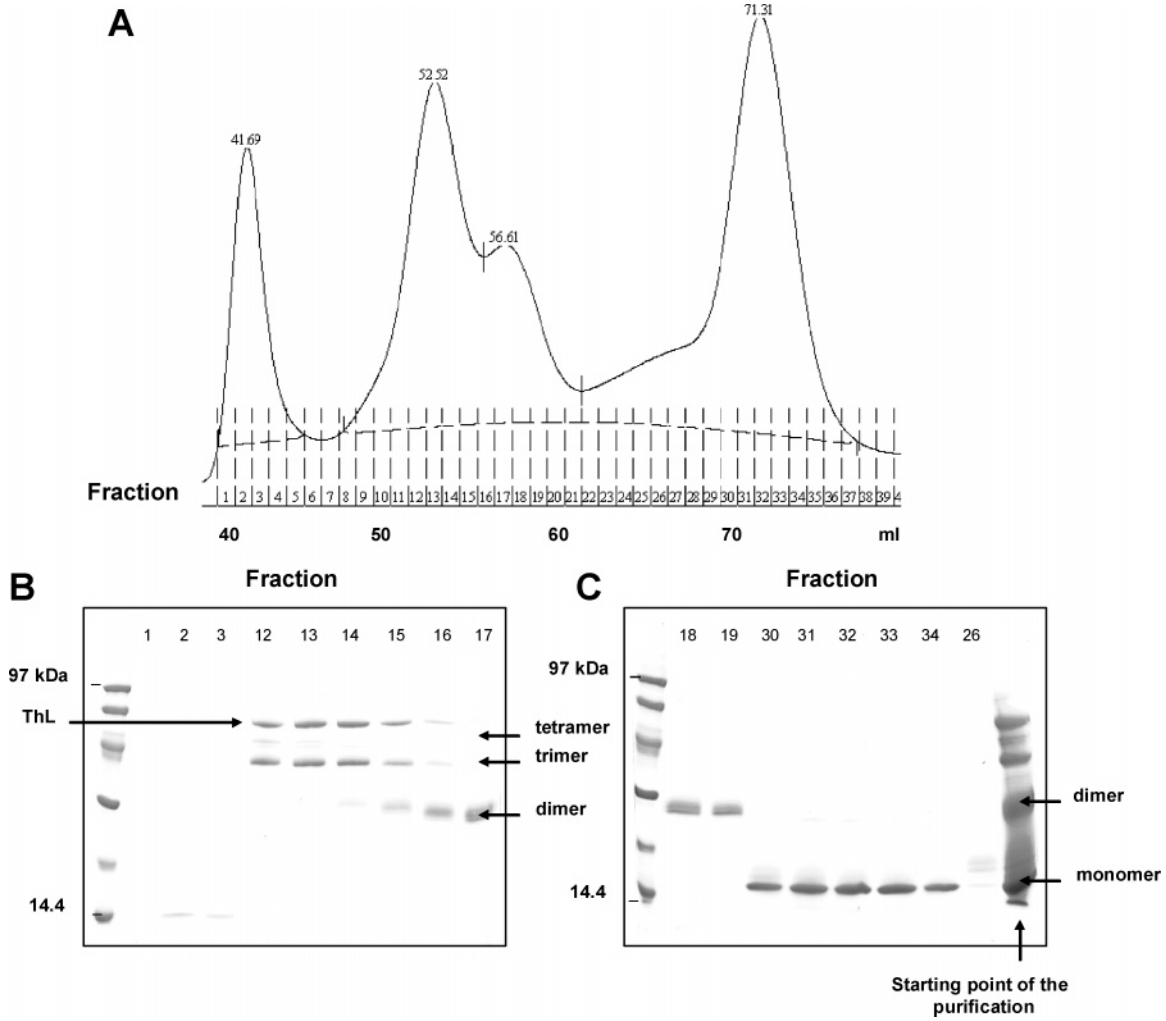


Figure 6. Separation of the oligomers formed in ThL-catalyzed reactions with full-length coactosin. (**A**) Gel-filtration chromatogram and corresponding SDS-PAGEs (**B** and **C**) from the main fractions. In panels **B** and **C**, the first lanes are low molecular weight markers (94, 67, 43, 30, 20, and 14 kDa) and the lanes 1–3, 12–19, 26, and 30–34 are from the corresponding fractions of the gel-filtration chromatogram. A sample from the starting point of the purification is shown in the last lane of SDS-PAGE in panel **C**. Treatment conditions: volume 1 mL, buffer 10 mM succinic acid, pH 6.0 containing 1 mM DTT, substrate concentration 0.25 mM coactosin (4 mg of pure protein), ThL dosage 4000 nkat/μmol, reaction time 17 h.

mers as expected on the basis of the results shown in **Figure 4A**. ThL-catalyzed oligomerization of other proteins such as bovine serum albumin (BSA, 67 kDa) and bovine β-lactoglobulin (18 kDa) was also tested, but no oligomerization products were detected by SDS-PAGE (data not shown).

For further study of the oligomers of full-length coactosin, a larger amount of the protein was polymerized by ThL. Separation of the reaction products by gel-filtration and analysis of the resulting fractions by SDS-PAGE are shown in **Figure 5**. The results show that the monomers (**Figure 5C**, lanes 30−34) could be separated from the oligomers, which partially coeluted with each other and with the ThL (**Figure 5C**, lanes 12−19). The monomer (**Figure 5C**, fractions 32 and 33) and dimer (**Figure 5C**, fractions 18 and 19) fractions were further purified by reversed-phase chromatography, which resulted in pure monomer and dimer preparations as analyzed by SDS-PAGE (data not shown).The reversed-phase purified coactosin monomer and dimer were digested with trypsin, and the resulting peptides were subjected to direct MALDI-TOF peptide mass fingerprint analyses as well as separation by reversed-phase chromatography followed by MALDI-TOF MS analysis of the collected peptides. In the tryptic peptide mass fingerprint of the full-length coactosin, the C-terminal peptide (residues 116−126, m/z = 1081.43 Da) could be seen and also identified by MALDI-TOF/TOF fragment ion analysis. In the corresponding peptide mass fingerprint from the purified coactosin dimer, this peptide was completely missing (data not shown). Instead, the digest from the full-length coactosin dimer contained a peptide with a moleculas mass of 2161.86 Da (**Figure 7**), which corresponds to the calculated mass (m/z = 2161.85 Da) of a tyrosine cross-linked dimer of the C-terminal tryptic peptide. This peptide was poorly visible in the total mass fingerprint but could easily be detected in one of the late reversed-phase chromatography fractions from the tryptic digest (data not shown).

DISCUSSION

In this work, the ability of ThL to oxidize tyrosine side-chains in a small protein was investgated using full-length and truncated coactosin as model substrate proteins. The aim of the work was to demonstrate the importance of the accessibility of tyrosine side-chains in the cross-linking and oligomerization of proteins by laccase. In addition, the simpler tripeptide Gly-Leu-Tyr was used as a model substrate to prove unambiguously that ThL is capable of generating covalent bonds between the aromatic rings of tyrosines.

On the basis of the data presented in this work, ThL could directly oxidize the small full-length coactosin protein without

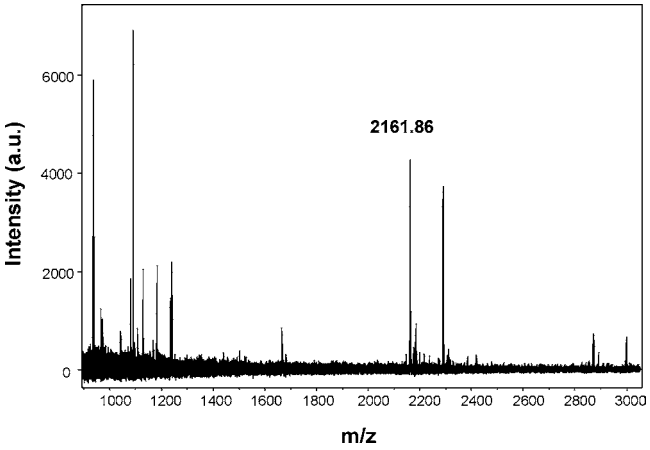
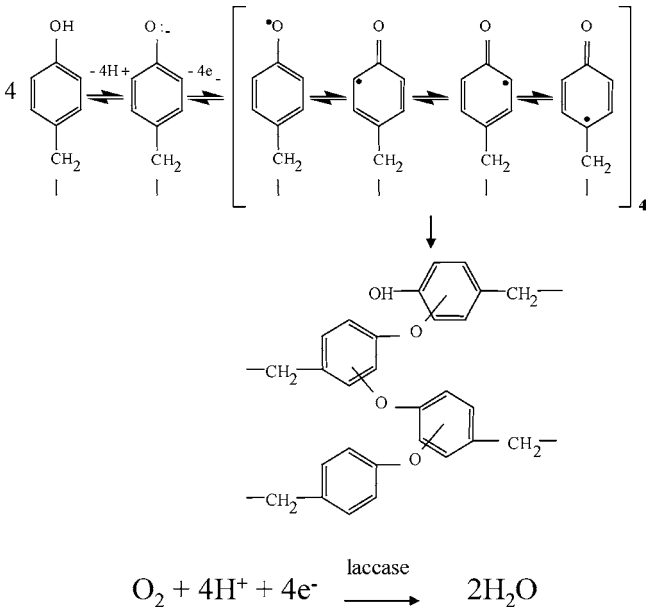


Figure 7. MALDI-TOF mass spectrum of a selected tryptic peptide fraction from purified and digested full-length coactosin dimer. The fraction contained the potential dimeric C-terminal peptide (m/z = 2161.86 Da), which cross-links two full-length coactosin monomers to the dimer. For analysis, the coactosin dimer, purified by gel-filtration and subsequent reversed-phase chromatography, was cleaved with trypsin, and the peptides formed were separated by reversed-phase chromatography followed by MALDI-TOF MS analysis of the fractions.

any auxiliary substances or mediators, although earlier studies with high molecular weight proteins suggested that small molecule mediators are necessary for protein cross-linking with laccases (*53*). Coactosin dimers, trimers, and tetramers were formed in the reactions with full-length coactosin, whereas no oligomerization products were observed with the truncated protein lacking the tyrosine-containing C-terminus. These findings support the conclusion that long flexible tails such as the C-terminal tail in the full-length coactosin are required for cross-linking of proteins by ThL. BSA and β-lactoglobulin lacking such flexible tails were not cross-linked by ThL in our studies. The covalent bonding between tyrosine residues in ThL-catalyzed oxidation was unambiguously proved by MALDI-TOF/TOF MS analysis of the trimer of the tripeptide Gly-Leu-Tyr. The covalent bonds formed between the tyrosine side-chains (i.e., via the aromatic rings of the tyrosines) were more stable than the ordinary peptide bonds since in laser-induced dissociation, Gly and Leu fragment ions could be sequentially removed from the molecule whereas the Tyr fragments remained bound to each other in the fragmentation conditions used.

β-Lactoglobulin has been extensively studied during recent years (*54*−*58*), and several 3-D structures of the protein have been determined by means of X-ray crystallography (*59*−*61*) and NMR spectroscopy (*62*−*64*). β-Lactoglobulin contains five tyrosine residues, only one of which is located on the surface of the protein. However, the overall shape of the β-lactoglobulin is highly globular, and the rotating aromatic ring of the tyrosine residue pointing outward from the surface of the protein is not accessible enough for enzymatic oxidation and subsequent oligomerization as is the tyrosine side-chain in the C-terminal tail of the full-length coactosin. In coactosin, most of the reactive tyrosines are located inside the protein, and their enzymatic accessibility is poor. This also supports the conclusion that in ThL-catalyzed reactions, cross-links were formed between tyrosines in the C-terminal tail. The structure of BSA is heart-shaped (*65*, *66*), and it contains 21 tyrosine residues (*67*−*71*). Despite the large number of tyrosine residues, the 3-D structure of BSA is also too compact for enzymatic oxidation of the tyrosine residues and subsequent oligomerization of the protein, as was the case with truncated coactosin. On the other hand, it has been reported that when using a salt-soluble chicken-breast

Scheme 1



myofibril protein mixture as the ThL substrate, the myosin heavy chain (MHC, 200 kDa) and troponin T (30 kDa) were the most affected proteins after ThL treatment (*43*). However, in that study, the substrate solution contained a number of other compounds in addition to the different proteins, which made it impossible to draw any conclusions about the reaction mechanisms of ThL-catalyzed oxidation of proteins.

It can be concluded that proteins, in general, are poor substrates for laccases. Only tyrosine, cysteine, and tryptophan residues could be oxidized by laccase. Protein folding (i.e., the accessibility of reactive amino acid side-chains) was the main factor determining the extent of protein cross-linking. The size of the protein also affected the degree of polymerization. Thus, ThL-catalyzed oligomerization of the full-length coactosin protein is expected to proceed by the same reaction mechanism (**Scheme 1**) as shown recently for short peptides (*27*).

ABBREVIATIONS USED

BSA, bovine serum albumin; Cys, cysteine; 3-D, three-dimensional structure; DTT, dithiothreitol; Gly, glysine; Gly-Leu-Tyr, glysine-leucine-tyrosine tripeptide; HPLC, high-performance liquid chromatography; Leu, leucine; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of flight mass spectrometry; MCBP, multicopper blue protein; MOC, multicopper oxidase; RP, reversed-phase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TFA, trifluoro acetic acid; ThL, *Trametes hirsuta* laccase; Trp, tryptophan; Tyr, tyrosine.

ACKNOWLEDGMENT

We thank Elina Ahola, Gunilla Rönnholm, and Heljä Heikkinen for their skillful technical assistance.

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Received for review August 21, 2006. Revised manuscript received September 20, 2006. Accepted September 21, 2006.

JF062397H