Laccase-Catalyzed Polymerization of Two Phenolic Compounds Studied by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight and Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry with Collision-Induced Dissociation Experiments

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Enzymatic oxidation of two phenolic compounds [syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) and 2,6-dimethylphenol] was studied. The products of laccase- and laccase−mediator-catalyzed oxidation reactions were monitored by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and further analyzed by electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) MS with collision-induced dissociation (CID) experiments. For the oligomers of syringic acid, some variability was observed in MALDI-TOF analysis. However, the origin of this variability could not be resolved on the basis of MALDI-TOF spectra due to the poor resolution of the instrument in use. The strength of ESI-FTICR MS was the high-resolution data provided from oligomers of syringic acid. The CID experiments were extremely useful for structural studies of oligomers and verified that the variability of the products was due to the end groups; the phenolic hydroxyl group was modified during the oxidation.

1. Introduction

In vitro synthesis of polymers via enzymatic catalysis has been extensively studied and developed.[1−3] Advantages of enzymatic synthesis of organic compounds include catalysis under mild reaction conditions (temperature, pressure, and pH), stereospecific reactions, and the "green" nature of enzymes as catalysts. Many useful polymers, difficult to manufacture by conventional methods, can be produced via enzymatic catalysis. Laccases (EC 1.10.3.2) are one of the most promising groups of enzymes for industrial purposes.[4,5]

Laccases are polyphenol oxidases belonging to the group of blue multicopper proteins, which as oxidoreductive enzymes perform the reduction of oxygen to water while at the same time oxidizing the substrate. The reducing substrate spectrum of laccase is exceedingly broad. Laccases catalyze the oxidation of inorganic and organic metal complexes, anilines, thiols, and especially phenols, providing their redox potentials are sufficiently low.[4,6] The catalysis of lignin degradation by laccases is also based on oxidation; degradation starts with the oxidation of phenolic components and the production of phenoxy radicals.[7] The exact role and importance of laccases in lignin degradation remain unclear, however. The industrial use of laccases in pulp manufacturing is of particular interest. The function of laccases in plants seems to be in contrast to the function in fungi; in plants they are a part of the lignin synthesizing system.[8]

As polyphenol oxidases, laccases mainly catalyze the polymerization of phenolic moieties. Thus, concurrently with the degradation of lignin, they may cause the lower molecular weight products to form polymeric compounds.[7] Laccase-catalyzed polymerization of phenols has been reported to lead to poly(phenylene oxide)s (PPOs).[9−11] Formation of PPO from syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) has been achieved with oxidoreductases, soybean and *Coprinus cinerius* peroxidases, and *Pycnoporus coccineus* and *Myceliophthore* laccases. The polymers were reported to form exclusively from 1,4-oxyphenylene units, and the terminal structure was described as containing a carboxylic acid group at one end and a phenolic hydroxyl group at the other.[9]

Laccases can also be used for the transformation of nonphenolic compounds, but not without mediator compounds.[12] For example, benzyl alcohols can be converted to benzaldehydes with a laccase−mediator system (LMS).[13,14] The influence of LMS on reactions of phenolic model compounds has been studied by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.[15] The polymerizing action of laccase without mediator and the depolymerization action of LMS were verified; LMS did not prevent the formation of polymers at the outset, but it degraded polymers after their formation.

Mass spectrometry (MS) has become an important technique for the analysis of polymer samples.[16,17] Detailed information is provided about polymers, complementary to information obtained by traditional techniques (i.e., gel-permeation chromatography, light scattering, osmometry, nuclear magnetic spectroscopy, and infrared spectroscopy), which describe the chemical functionality of the repeat units and sometimes the end groups.

This paper reports on the mass spectrometric characterization of enzymatically prepared polymers of syringic acid and 2,6-dimethylphenol. Spectra measured by MALDI-TOF MS contained peaks indicating variability for the oligomers and polymers produced with laccase and LMS. The observed variability was in disagreement with the findings of earlier investigations and could not be resolved on the basis of the MALDI spectra. Electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometer, with its

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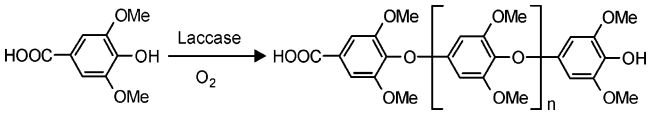
Scheme 1



better resolving power than the MALDI-TOF system, was utilized to clarify the structure of the oligomers. Accurate mass measurements and collision-induced dissociation (CID) experiments made with the ESI-FTICR instrument revealed the source of the variability in the oligomers of syringic acid.

2. Experimental Section

2.1. Materials. All compounds were commercially available and were used as received. The laccase from *Trametes versicolor* (Tv, also known as *Coriolus versicolor*), was used in polymerization reactions. The enzyme was furnished by Sigma−Aldrich and two different lots were used, with activities of 24 and 0.83 units/mg.

2.2. Laccase-Catalyzed Polymerization. Laccase-catalyzed polymerization was usually carried out as follows: Monomer (2,6-dimethylphenol (0.31 g, 2.5 mmol) or syringic acid (0.50 g, 2.5 mmol)) was dissolved in organic solvent (usually acetone, 10 mL). Ammonium acetate buffer was added (15 mL, pH 5.0, 0.1 M), followed by laccase (2.95 mg) dissolved in 250 μL of ammonium acetate buffer. The lower activity of the second lot of Tv laccase (0.83 unit/mg) was compensated by using a larger amount of the enzyme. The mixture was stirred under air for a predetermined time (up to 24 h). The purification steps were much the same for the polymers of 2,6-dimethylphenol and syringic acid. After the reaction, the polymerization mixture of 2,6-dimethylphenol was filtered and washed with water and methanol. Finally the solid was dissolved in a small amount of chloroform and precipitated with a large amount of methanol. In the case of syringic acid, the solvent from the polymerization mixture was evaporated by rotary evaporator and the residue was washed with water and methanol. Reprecipitation of the material was done for a few samples but was later omitted as it had no influence on the spectra, and the yields were better without this step. Typical yields were 40−50% for polymers of 2,6-dimethylphenol and 54−58% for polymers of syringic acid. The conditions of polymerization were varied by using different compositions of the solvent and different reaction times. The usual reaction time was 24 h. Typical characteristics of polymers with that reaction time were, for syringic acid, number-average molecular mass (M_n) 1172 Da, weight-average molecular mass (M_w) 1444 Da (molecular mass distribution range ca. 600−3400 Da); and for 2,6-dimethylphenol M_n 1553 Da, M_w 2013 Da (molecular mass distribution range ca. 400−3300 Da). Also, two mediators [2,2′,6,6′-tetramethylpiperidine-*N*-oxyl radical, TEMPO, and 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid, ABTS] were tested with laccase in the polymerization of syringic acid. The amount used was 0.25 mmol.

2.3. Mass Spectrometry. Mass spectrometric measurements were performed with a Bruker Proflex MALDI-TOF mass spectrometer and with a Bruker BioApex II 47e FTICR mass spectrometer equipped with an external Bruker Apollo ESI ion source. The Bruker FTICR instrument contains an infinity cell and a 4.7 T superconducting magnet. With the electrospray source in operation, a base pressure of 1.0×10^{-9} Torr (1 Torr = 133.3 Pa) was achieved in the cell. Liquid samples were introduced through a syringe infusion pump at a flow rate of 1500 nL/min. The normal parameters used in the measurements were as follows: nitrogen was used as drying gas (100 °C), the ion source capillary voltage was −4.3 kV, and the capillary exit voltage at the end of the glass capillary was set at 200 V. Data collection and processing were performed with XMASS 6.0.2 software. Each spectrum was an average of 32 scans. Calibration of the *m*/*z* region was made externally with a water−acetonitrile solution of sodium trifluoroacetate.[18] In CID experiments, argon was used as both cooling and collision gas. Ions generated in the external ion source and transferred to the cell were collisionally cooled with argon, which was introduced to the cell through a leak valve. After a delay of 1 s the cooled ions were isolated by the correlated harmonic excitation field (CHEF) technique.[19] Isolated ions were translationally excited by an on-resonance radio frequency pulse, and another gas pulse of argon was introduced. The excited ions were allowed to collide and dissociate with argon during 5 s before the product ion frequency sweep.

The Bruker Proflex instrument is equipped with a nitrogen laser (λ = 337 nm) and operated in linear mode with acceleration voltage of 20 kV. Usually 100 laser shots were used to produce one spectrum. Dithranol [1,8-dihydroxy-9(10*H*)anthracenone] was used as a matrix

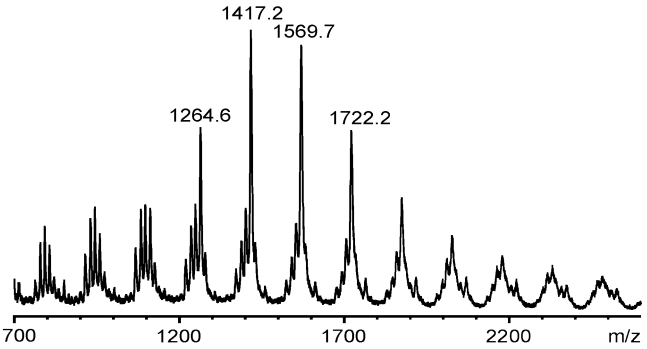


Figure 1. MALDI-TOF mass spectrum of syringic acid after 24 h of reaction time with Tv laccase.

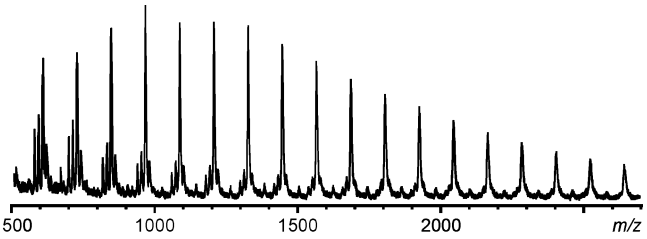


Figure 2. MALDI-TOF mass spectrum of 2,6-dimethylphenol after 24 h of reaction with Tv laccase.

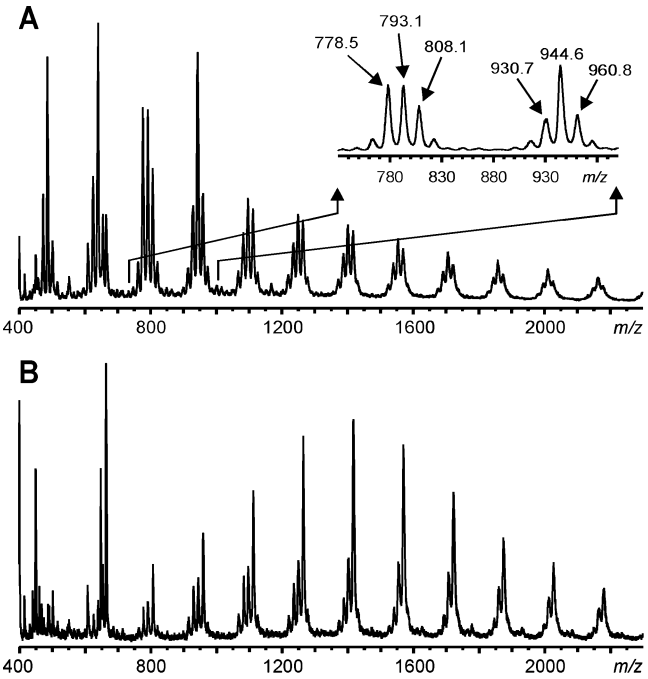


Figure 3. MALDI-TOF mass spectra of syringic acid after 2 h of reaction time. Reactions contained (A) 2.5 mmol of syringic acid, 2.95 mg of Tv laccase, and 0.25 mM mediator TEMPO or (B) 2.5 mmol of syringic acid and 2.95 mg of laccase.

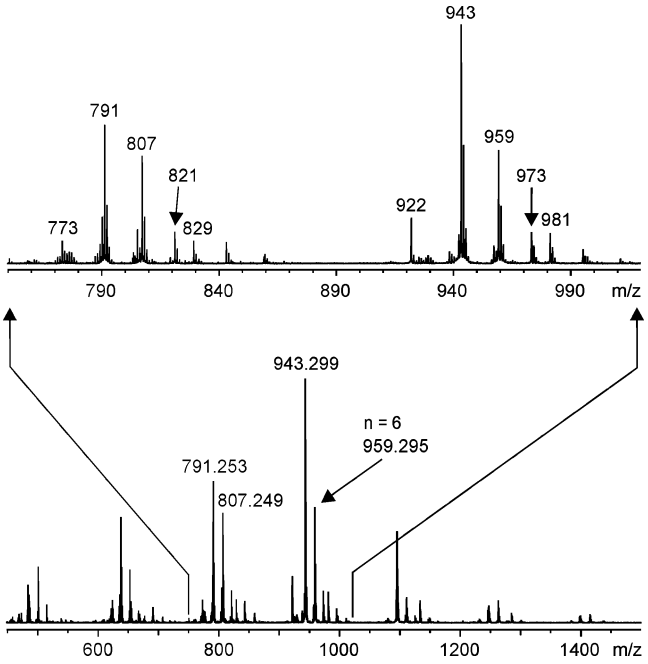


Figure 4. ESI−FTICR mass spectrum of syringic acid after 2 h of reaction time with Tv laccase and TEMPO. *n* indicates the number of monomers. The enlargement shows the heterogeneity of the peaks.

Table 1. Theoretical and Experimental Masses of Different Kinds of Oligomers Formed in LMS-Catalyzed Polymerization Reaction of Syringic Acid[a]

no. of	mass of M + H^+ (Da)		mass of M + Na^+ (Da)		smaller mass form (Da)
monomers	theor	exptl	theor	exptl	exptl
5	807.249	807.249	829.231	829.233	791.253
6	959.296	959.295	981.278	981.279	943.299
7	1111.343	1111.338	1133.325	1133.330	1095.346

[a] See also the spectrum in Figure 4. Suggested structures for the oligomers are presented in Figure 5 (smaller mass form has hydrogen instead of hydroxyl in the end group).

(dissolved in chloroform, 20 mg/mL). Samples were dissolved in chloroform (10 mg/mL) and the sample/matrix mixing ratio was 2.5 μL/10 μL.

3. Results and Discussion

Earlier investigations have shown the potential of laccases in the polymerization of phenolic compounds containing different side groups. These useful new strategies can lead to polymers that are difficult to prepare by traditional polymerization reactions. While studying methods for laccase-catalyzed polymerization, we encountered some discrepancies with earlier investigations in the field. In the present study, laccase was employed for the polymerization of syringic acid and 2,6-dimethylphenol.

3.1. MALDI-TOF Measurements of Laccase-Catalyzed Reactions. The formation and nature of polymers from laccase-catalyzed reactions were first investigated by MALDI-TOF MS. Several matrixes [2-(4-hydroxyphenylazo)benzoic acid (HABA), 2,5-dihydroxybenzoic acid (DHB), 3,5-dimethoxy-4-hydroxycinnaminic acid (sinapic acid, SA), and 1,8-dihydroxy-9(10*H*)-anthracenone (dithranol)] in different concentrations were tested for their suitability, and dithranol was found to produce the best ionization. As mentioned above, laccase-catalyzed polymerization of syringic acid has been reported to lead to polymers

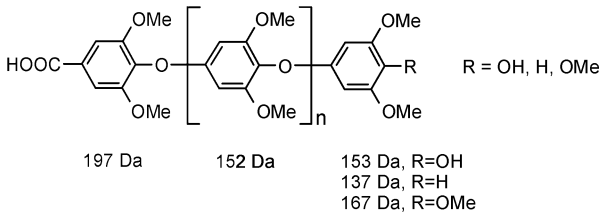


Figure 5. Structures of different kinds of polymers constructed from syringic acid.

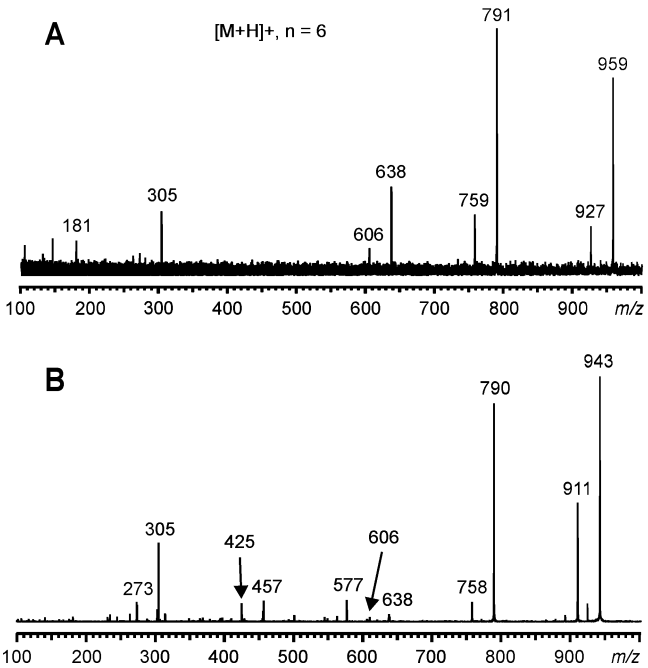
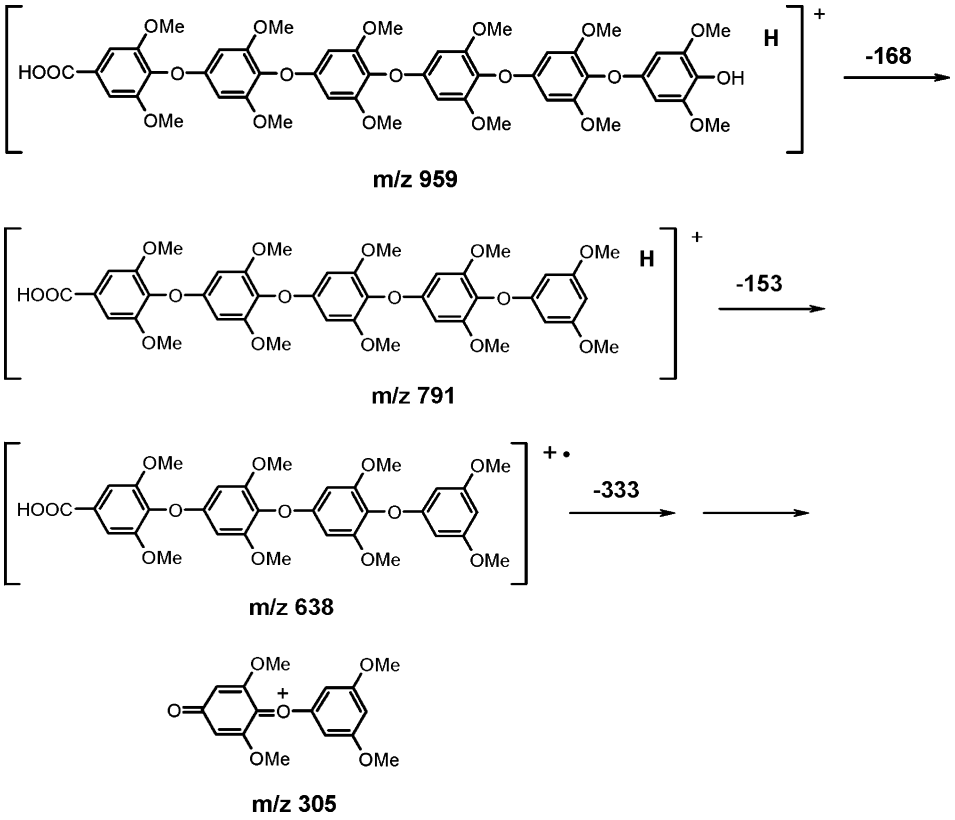


Figure 6. CID mass spectra of two different ions observed in the laccase-catalyzed polymerization reaction: (A) the protonated hexamer (*m*/*z* 959) of syringic acid containing benzoic acid and phenolic hydroxyl end groups and (B) the protonated hexamer of syringic acid with 16 Da smaller mass (*m*/*z* 943) than the ordinary hexamer.

consisting exclusively of 1,4-oxyphenylene units, with a benzoic acid group at one end of the polymer chain and a phenolic group at the other[2,9] (see Scheme 1).

Figure 1 shows a MALDI-TOF mass spectrum measured from a Tv laccase-catalyzed polymerization mixture of syringic acid. The reaction was carried out with similar amounts of laccase and monomer as in earlier work on the same polymerization system.[9] As can be seen in Figure 1, the MALDI spectrum of the formed polymer is characterized by a series of peak groups representing oligomers of different length. The main peaks of the successive groups are separated by 152.5 Da, corresponding to the mass of one 1,4-oxyphenylene unit, and they match with the masses of protonated species with $[nM + H]^+$ (where *n* is the number of monomeric units and the end groups are benzoic acid and phenolic group). The other, smaller peaks, which appear systematically in all spectra (measured for reaction conditions differing in amounts of organic solvent and buffer and temperatures), indicate variability in the formed oligomers. Some of the smaller peaks in each peak group are in a lower *m*/*z* region than the peaks of the protonated species and could not, therefore, have arisen from adducts such as sodium or potassium, which give rise to peaks in a higher *m*/*z* region than those of protonated species. The origin of these additional peaks could not be identified on the basis of the mass spectra measured by MALDI-TOF MS because the resolving power and mass accuracy are too low.

Scheme 2

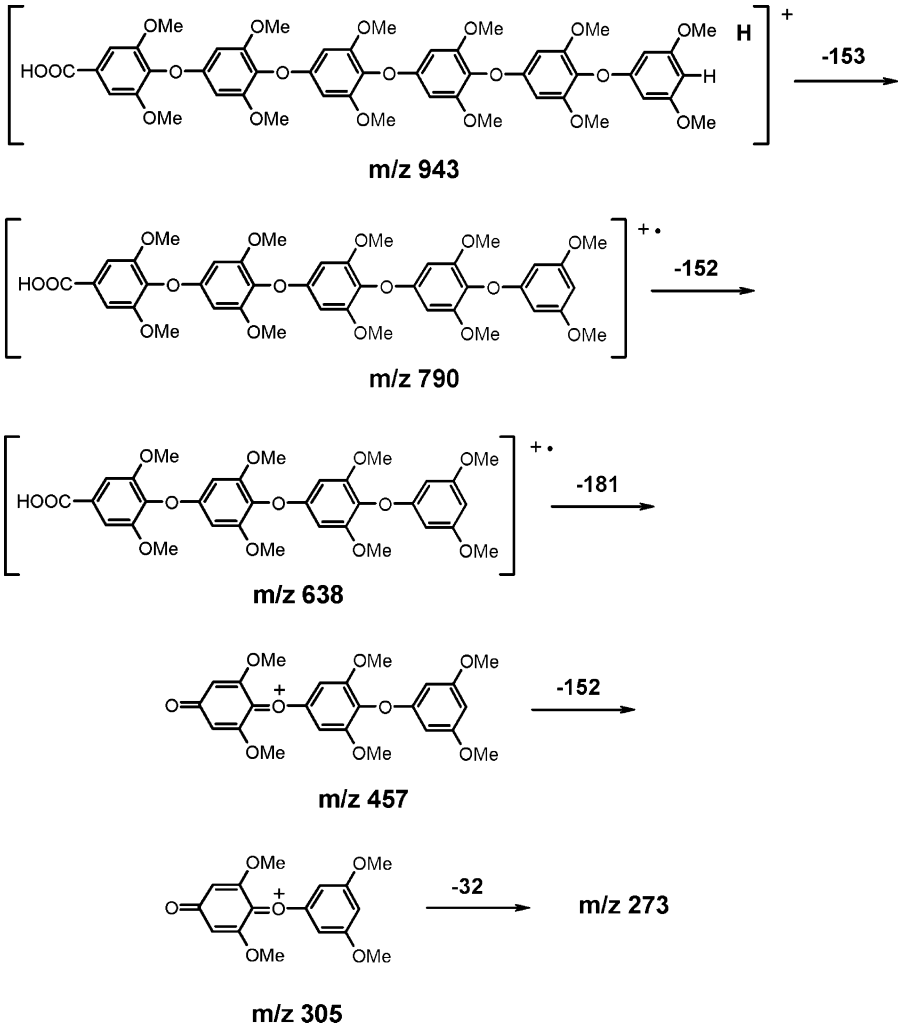


Laccase-catalyzed oxidation of 2,6-dimethylphenol has been reported to yield poly(2,6-dimethyl-1,4-oxyphenylene).[10] The mass spectrum we measured from laccase-catalyzed polymerization of 2,6-dimethylphenol is presented in Figure 2. Peaks in the lower *m*/*z* region show a pattern similar to the peaks observed for syringic acid and indicate that the oligomers of 2,6-dimethylphenol likewise contain variability in their structures.

3.2. MALDI-TOF Measurements of LMS-Catalyzed Reactions. Simultaneously with the normal laccase-catalyzed polymerization reactions, we carried out oxidation reactions of syringic acid with a laccase−mediator system. Figure 3 shows the mass spectrum of the product oligomers of syringic acid with LMS (mediator 2,2′,6,6′-tetramethylpiperidine-*N*-oxyl radical, TEMPO) after 2 h of reaction time. [The spectrum obtained with use of another mediator, 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), was similar; data not shown.] The mass spectrum measured from the normal laccase-catalyzed reaction mixture after the same reaction time is included for comparison. The effect of mediator on the oligomers formed from syringic acid is considerable. The oligomeric peaks of greatest intensity appear at much lower *m*/*z* region than those for normal laccase-catalyzed reactions. The base peak in the spectrum of the LMS-catalyzed reaction arises from a form consisting of four monomeric units. In contrast, the normal laccase-catalyzed polymerization, after 2 h of reaction time, yielded a base peak attributable to a form consisting of nine monomeric units. The other notable feature of the LMS-catalyzed polymerization reactions is the increased intensities of the additional peaks indicating the more abundant formation of species with variation in their structure. The most likely explanation for the additional peaks is variation in the terminal structures, that is, in the benzoic acid or phenolic groups. The first step in the proposed mechanism of laccase-catalyzed polymerization of syringic acid is hydrogen abstraction from the phenolic group,[2,9] producing phenoxy radicals, which are further coupled with each other to give a quinoid-type intermediate. With this reaction mechanism the variation of the terminal structure presumably is focused at the phenolic group, while the benzoic acid group remains unchanged. The mass differences of the fine structure peaks in the MALDI spectra range between 14 and 20 Da (see enlargement of Figure 3). MALDI spectra measured from the laccase-catalyzed polymerization mixture of 2,6-dimethylphenol support terminal structure variation at the phenolic end groups, since fine structure peaks were present despite the lack of the benzoic acid group.

3.3. ESI-FTICR Measurements. ESI-FTICR mass spectrometric measurements were made to clarify the origin of the peaks in MALDI spectra that indicated variation in end groups. The advantage of this instrument is its better resolving power and mass accuracy compared with MALDI-TOF, as well as the capability for collision-induced dissociation (CID) experiments, which are useful for determining the structures of oligomers. The ESI-FTICR mass spectrum measured from the LMS-catalyzed polymerization reaction mixture of syringic acid is shown in Figure 4 (oligomers of 2,6-dimethylphenol did not give a signal by ESI). The mass values are presented in Table 1, and the experimental molecular masses (monoisotopic accurate masses) confirmed that the peaks arise from oligomers containing benzoic acid and phenolic hydroxyl end groups. (The same sample was measured with MALDI; see Figure 3.) Oligomers of syringic acid were detected as protonated species, as with MALDI, and the peaks indicating variability in the oligomers were also present in the ESI spectrum. The oligomers containing benzoic acid and phenolic hydroxyl end groups did not produce the most abundant peaks in the oligomer distribution produced by LMS-catalyzed polymerization (see Figure 4). The mass difference between protonated species and the species with highest intensity was slightly less than 16 Da, which corresponds to the monoisotopic mass of an oxygen atom (see Table 1).

Scheme 3



ESI-FTICR mass spectrometric measurements were also performed for the laccase-catalyzed polymerization of syringic acid. The recorded spectrum (not shown) contained fewer peaks, indicating a smaller number of oligomer variants than in the LMS-catalyzed reactions. This was in agreement with the results obtained by MALDI-TOF MS.

Inspection of both MALDI and ESI mass spectra shows that, in addition to the peaks already mentioned, most peak groups also contain peaks in higher and lower *m*/*z* regions than the two main forms. One of these minor forms has 14.016 Da larger mass than the protonated hexamer (this form was also seen for other oligomers of different length) arising from syringic acid, which contains hydroxyl at one end of the chain. This mass difference matches well with the theoretical mass of the CH_2 group (methylene, 14.016 Da). The only oligomer that reasonably could produce this peak would contain an additional methoxy group in place of the hydroxyl group. The proposed structures of three different kinds of oligomer formed during laccase-catalyzed oxidation of syringic acid are presented in Figure 5. Laccase induces radical reactions, which are not very specific reactions. The radical site can move and locate in different places (intermediates). These intermediates can further react together, producing variable products. This must be the main reason for the observed heterogeneity.

The enlargement of the ESI-FTICR mass spectrum in Figure 4 reveals that there is, in fact, some further heterogeneity in the oligomers (more than indicated by the MALDI spectra). The peaks representing these oligomers vary in height, and their intensities are low compared with the intensities of the main forms (*m*/*z* 805, for example, which is of 2 Da smaller mass than the protonated pentamer, *m*/*z* 807). The mass spectrometric investigations do not allow a conclusion about the origin of these peaks.

3.4. CID Experiments. The origin of two peaks arising from syringic acid, the protonated hexamer (containing benzoic acid and phenolic end groups) and the peak with 16 Da smaller molecular mass, was studied by CID experiments. The CID spectra of these two ions are presented in Figure 6, and the proposed structures and proposed fragmentation pathways leading to the spectra are in Schemes 2 and 3. The most intense ion after CID of the protonated hexamer was the ion at *m*/*z* 791 (Figure 6A, Scheme 2). The only possible fragmentation leading to this ion starts from the side of the phenolic group of the hexamer, and the first monomeric unit is cleaved. The cleavage occurs between the carbon−oxygen bond of the oligomer backbone, and the cleaved neutral (168 Da) most likely has a quinoid-type structure. Further dissociation occurs along the backbone as shown in Scheme 2. Many fragmentations of 32 Da were observed (from ions at *m*/*z* 959, 791, and 638). This mass corresponds to the mass of methanol and must represent the loss of methoxy side groups. The oligomer has many methoxy groups, however, and it is not possible to say which of them were lost. Note that the dissociation pathways in Schemes 2 and 3 are proposed pathways, and the origin of the fragments was not confirmed by multiple MS (MS/MS/MS) experiments.

The mass difference between separate oligomers corresponding to oxygen, and the radical mechanism for syringic acid polymerization, suggested variation on the phenolic side of the hexamer. The CID experiment supported that interpretation. The proposed structure and proposed dissociation pathway for the ion at *m*/*z* 943 are presented in Scheme 3. The cleavage of PPOs occurs from the carboxylic end side of each backbone ether bond, starting from the hydroxyl end side (see Scheme 2). The most intense fragment ion in the spectrum in Figure 6B is at *m*/*z* 790 and corresponds to the loss of 153 Da from the ion at *m*/*z* 943. Since the mass of 153 Da is the mass of the phenolic side end group without the phenolic oxygen atom, the ion at *m*/*z* 943 represents a hexamer with no phenolic group and only methoxy groups as substituents at that end of the chain. Further cleavages occur between the carbon−oxygen bonds of the oligomer backbone.

4. Conclusions

Two different mass spectrometric techniques were used for the study of the laccase-catalyzed polymerization of two phenolic compounds. By both of these techniques (MALDI-TOF and ESI-FTICR MS), the oligomers of different lengths from syringic acid were observed as protonated species and sodium adducts. Also some species due to structural variability were observed. The origin of the variability was resolved on the basis of ESI-FTICR MS measurements and CID experiments. CID experiments allowed the step by step analysis of oligomer chains. The two main forms formed in the laccase-catalyzed polymerization of syringic acid were similar except for the end groups: one contained a hydroxyl group, while the other lacked this group and contained hydrogen instead. Yet one other form was observed for oligomers of different length polymerized from syringic acid; it, too, most likely varied in one end group, having a methoxy group instead of a phenolic hydroxyl group. The heterogeneity of the oligomers formed from syringic acid was observed for both laccase- and LMS-catalyzed polymerization. The LMS-catalyzed polymerization yielded oligomers with a lower degree of polymerization, and the formed oligomers also had more variation in their structures than oligomers constructed without mediator.

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