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Hardening Reaction of Alkenylcatechol Derivatives by Laccase from *Pycnopus Coccineus*

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Abstract

The relationship between the hardening reaction of alkenylcatechol derivatives by laccase and the stereo structure of the side chain double bond has been studied considering the former as an urushiol analogue. We synthesized several kinds of catechol derivatives with a C₁₈-alkenyl side chain at 4-position of the catechol ring. The side chain of these compounds have different numbers of double bonds having different configurations. The hardening reaction of these catechol derivatives by laccase from *Pycnopus coccineus* was carried out in the presence of polysaccharides and glycoproteins, in order to measure the drying time and the viscoelastic behavior of the dried films. Hardening was observed for the dienylcatechol derivative having *cis* double bonds at C-9', C-12' yielding the films which showed ideal dynamic viscoelasticity. On the other hand, such films were not obtained in the case of dienylcatechol having a mixture of geometrical isomers, 72% of which were *trans* isomers. We suggest that the methylene-interrupted *cis* double bond pair can be crosslinked to a greater extent than the *trans*-ones by the laccase.

Key-words : *Urushi, Hardening reaction, Alkenylcatechol derivative, Stereo structure, Laccase*

1. Introduction

The fluid sap exuded from several kinds Japanese or Chinese lac trees, *Rhus vernicifera*, dries into a tough and brilliant film and has been used as a naturally occurring coating material for thousands of years in the Orient¹⁾. It is a latex compound of urushiol congeners^{2~5)} (60%), water (30%), plant gums (polysaccharides)⁶⁾ (7%), water-insoluble glycoproteins and enzymes (laccase etc.)⁷⁾ (ca. 0.1%).

It is a very interesting task to develop a laquer paint as a substitutional model for urushi which is usually dried at room temperature with high humidity. The principal reaction of the film-formation process is believed to be oxidative coupling of urushiol under the catalytic action of the oxidoreductase laccase⁸⁾. It was demonstrated that the initial oxidative reaction course of the sap or lacquer formed from the sap involves a specific laccase-catalyzed oxidation of urushiol into the corresponding *o*-quinone and the

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subsequent reaction of the quinone thus formed with the remaining urushiol, another quinone, or other components of the sap or the lacquer^{9, 10}. The unsaturated side chain of the urushiol molecule plays an important role in the reaction. There have been several studies^{11~13} about the role of the unsaturated side chain in the hardening reaction of Japanese Urushi, but none of them has elucidated the steric effects.

Fig. 1 Structures of alkenylcatechol derivatives

We have attempted to obtain further insight into the hardening reaction by examining the reaction of alkenylcatechol derivatives with their side chain having different numbers of double bonds of different configurations. We synthesized such catechol derivatives with a C₁₈-alkenyl side chain at the 4-position of the catechol ring, examples being, a triene compound (trienylcatechol) having three *cis* double bonds at C-9', C-12', C-15', another (dienylcatechol I) of two dienes having two *cis* double bond at C-9', C-12', another (dienylcatechol II) with a mixture of three geometical isomers 72% of which are *trans*-isomers, and a monoene (monoenylcatechol) having *cis* double bond at C-9' (**Figure 1**). Recently it was found that *Pycnoporus coccineus* exuded a highly active laccase (laccase-*Pyc*)¹⁴, which was also commercially available. The hardening reaction of these catechol derivatives by laccase-*Pyc* was carried out in the presence of polysaccharides and glycoproteins derived from low-grade non-drying urushi, in order to measure the drying times and the viscoelastic behaviors of the dried films. The results obtained offers information as to the specific contribution of steric structure of the unsaturated side chain to the hardening process.

2. Experimental

2.1 Materials

2.1.1 Alkenylcatechol derivatives

The catechol derivatives were synthesized from unsaturated fatty acid by the same method as described by Terada¹⁵. Initially polybromide was produced by the addition of bromine to unsaturated fatty acid; next the polybromide was converted into the corresponding acid chloride, which was introduced to the veratrole ring by Friedel-Crafts substitution using anhydrous aluminum chloride. Next, demethylation of the methoxy group by boron tribromide followed by Clemmensen reduction to restore the double bond. These alkenyl side chains have different numbers of double bonds of different configuration respectively. The trienylcatechol from linolenic acid has three *cis* double bonds at 9, 12, 15-positions. In the case of linoleic acid, the dienylcatechol I of two dienes has two *cis* double bonds at 9, 12-positions and the another dienylcatechol II is a mixture of three geometical isomers, *trans-trans*, *trans-cis*, *cis-cis*. The monoenylcatechol from oleic acid has a *cis* double bond at the 9-position. These structures were identified by 270 MHz ¹H-NMR and IR spectra. The configurations of the side chain double bonds

were determined by ^1H coupling experiments, which follow the separation by the high-performance liquid chromatography especially in the case of the dienylcatechol II.

2.1.2 Laccase from *Pycnoporus coccineus* (laccase-*Pyc*).

The enzyme was produced by the same method as reported by Oda¹⁶⁾. *Pycnoporus coccineus* was cultured in Oda's medium on standing at 25°C for 4 days, followed by the shaking the culture at 25°C for 2 weeks. The mycelia were removed by filtration from the culture medium. A highly active laccase, laccase-*Pyc*, was purified from the concentrate of the filtrate. Laccase-*Pyc* selectively oxidizes dihydroxy benzene and its derivatives. The aqueous enzyme solution exhibits blue color. The molecular weight is about 70,000, and the optimum reaction pH is 4.0 to 6.0. It is stable up to 60°C. The activity was assayed by the oxygen up-take method¹⁷⁾ using 0.1 M Na acetate buffer, pH 4.5, containing 5 mM catechol as substrate. The amount of protein was measured by the Lowry's method¹⁸⁾. The enzyme solution used for this experiment has an activity of 122,000 units per ml, and 1,900 units per mg protein.

2.1.3 Mixture of polysaccharides and glycoproteins

The mixture was obtained from non-drying urushi in China by the method described in the literature¹⁹⁾. Residual enzyme activity was measured in the mixture as described above, and it was found that active laccase was not present.

2.2 Hardening reaction

The hardening procedure was as follows. 0.10 grams of a mixture of polysaccharides and glycoproteins was added into various activities of laccase-*Pyc* (6.10×10^4 to 2.44×10^5 unit), followed by the required mixing. Then 1 gram of the alkenylcatechol derivative was added into the mixture. Mixing was continued until the sample water content reached about 25%. The artificial urushi samples coated on glass plates were immediately set to harden in the thermohygrostat set up for 30°C, 80% RH.

2.3 Measurements

The analytical high performance liquid chromatography equipment (Tosoh Co. Ltd.) for the alkenylcatechol derivatives consisted of a CCPD pump, a Reodine sample injector valve and a RI-8010 refractive index detection. Peak retention times and areas were determined with a SC-8010 data system (Tosoh Co. Ltd.). Stainless steel column (15 cm $\times$ 4.6 mm I.D.) were slurry packed with ODS-silica gel (Wakosil-II 5 C 18 HG, 5 μm ; Wako pure chemical industry Co. Ltd.) for reverse phase column. The chromatography was carried out under the following conditions: volume injected, 20 μl ; column temperature, 35°C; flow-rate, 1.0 ml/min. All samples were injected as 1% solutions in acetonitrile with an elution of acetonitrile: water (70:10, v/v).

The artificial urushi samples were coated on glass plates (2.5 $\times$ 35 cm) by using a film applicator (slit thickness 37.5 μm). An RCI type drying time recorder installed in the thermohygrostat set up for 30°C, 80% RH. Drying time was determined by reading the time when a scratch had completely disappeared on the coated samples.

FT-IR spectra were recorded at regular intervals on a FT-IR 1760 (Perkin Elmer Co., Ltd.) at 4 cm^{-1} resolution with the total of 64 scans from 400 to 4,000 cm^{-1} . The samples were coated on KRS-5 (the mixed crystal with TII: TIBr molar ratio of 1:1) and set to

harden in the thermohygrostat set up for 30°C, 80% RH.

After the samples were coated over glass plates (7×15 cm) with an applicator (slit thickness 50 μ m), they were hardened at 30°C, 80% RH to form artificial urushi films with a thickness of about 30 μ m. We measured the temperature dependance of the dynamic viscoelasticity of the cut films (3×28 mm) with a Reovibron DDV-II-EP (Orientic Co. Ltd.). All measurements were carried out under temperatures from 20 to 200°C, frequency 3.5 Hz and rate of temperature rise 1°C/min.

3. Results and Discussion

Figure 2 shows the high-performance chromatograms of the alkenylcatechol derivatives. The chromatographic system (see experimental section) was found to be effective for the separation of monoalkenylcatechol, dialkenylcatechol I, II and trialkenylcatechol. The trialkenylcatechol had a main retention time peak at 8.6 minutes along with a small one at 9.0 minutes. The dialkenylcatechol II had three peaks in the range of 11.7 to 13.1 minutes, with the 11.7 minutes peak overlapping the dialkenylcatechol I. The monoalkenylcatechol had the latest peak at 21.1 minutes. Monoalkenylcatechol and dialkenylcatechol I form a single peak each, and trialkenylcatechol is about the same. Thus they are singular compounds having almost *cis*-configuration in the side chain. On the other hand dialkenylcatechol II is a mixture of geometrical isomers with *trans-trans* : *trans-cis* : *cis-cis* area ratio of 28 : 44 : 28.

Hardening reactions of these alkenylcatechols by laccase-*Pyc* were carried out in the presence of polysaccharides and glycoproteins derived from non-drying urushi. **Table 1** shows the formulation and drying time of the artificial urushi samples. Hardening was

Fig. 2 High-performance liquid chromatograms of alkenylcatechol derivatives

Fig. 3 Changes in IR spectra of film hardened with time

Table 1 Formulation and drying time of the artificial urushi samples

Abbreviation	1	2	3	4	5	6
Trienylcatechol	1.00	—	—	—	—	—
Dienylcatechol I	—	1.00	—	—	—	—
Dienylcatechol II	—	—	1.00	1.00	—	—
Monoenylcatechol (g)	—	—	—	—	1.00	1.00
laccase- <i>pyc.</i> (μ l)	1000	1000	1000	1500	1000	1500
Specific activity ($\times 10$ unit/g)*	1.22	1.22	1.22	1.83	1.22	1.83
Aceton powder (g)	0.10	←	←	←	←	←
Drying time (hr.)**	12.0	13.0	×	120	×	×

* alkenylcatechol derivatives

** × represents no hardening

observed only in trienylcatechol and dienylcatechol I, after about 13 hours using 122,000 units laccase per gram the alkenylcatechol, yielded excellent films resembling natural urushi film. No film was formed with dienylcatechol II having three geometric isomers. However, a very soft film was formed after 120 hours by using much more laccase. Monoenylcatechol seemed not to form film even when a large excess of laccase was added. Thus, we suggest that methylene-interrupted *cis-cis* double bonds contribute much more to the hardening reaction than *trans-trans* or *trans-cis* ones.

Figure 3 shows the IR spectral change of the artificial urushi sample of dienylcatechol I, the hardening of which yielded excellent film using 122,000 units laccase-*Pyc* per gram of the alkenylcatechol. From this figure it can be seen that the tendency for the IR spectral change is similar to that of a normal sugurome-urushi²⁰. The absorption at 1652 cm^{-1} which is characteristic of the quinone, firstly appeared and then its intensity gradually decreased with time, suggesting a contribution of the quinone to the film-formation. The oxidative polymerization of the side chains is revealed in the dried film by the distinct decrease of absorption at 3010 and 723 cm^{-1} , which are attributable to $=\text{C-H}$ stretch vibration and $=\text{C-H}$ deformation vibration out of plane respectively. In addition, the increase of the C=O absorption observed at 1723 cm^{-1} was presumably due to oxidation of the side chains. Benzene ring substitution bands for dienylcatechol I were observed between $870\sim 780\text{ cm}^{-1}$. The absorption of a single hydrogen in the benzene ring at 870 cm^{-1} increased in conjunction with a decrease in the absorption of two adjacent hydrogen atoms in the ring at around 800 cm^{-1} . In addition, the absorption at 1114 cm^{-1} which is attributable to the ring hydrogen rocking vibration decreased. It indicates that formation of bonds at the 5 or 6 position of dienylcatechol I causes the disappearance of adjacent atom pairs, and the appearance of single atoms.

Figure 4 shows the relationship between the total activities of added laccase-*Pyc* and drying speed of the artificial urushi sample using dienylcatechol I. The speed, the reciprocal of the drying time, was plotted as a function of the activity on semilogarithmic graph paper. It was observed that the logarithmic drying speed linearly increased with the increase of total enzyme activities. Thus we can confirm the participation of the

Fig. 4 Relation between drying speed and total activities of laccase-*Pyc*

Fig. 5 Dynamic viscoelasticity of artificial films

enzyme in hardening reaction of the alkenylcatechols.

Figure 5 shows the dynamic viscoelasticity of artificial films. Two kinds of films obtained from dienylcatechol I and trienylcatechol using 122,000 units laccase-*Pyc* per gram alkenylcatechol were tested. We measured the temperature dependence of the dynamic viscoelasticity of films after 30 days drying. These artificial urushi films showed gently sloping E' -temperature curves and $\tan \delta$ -temperature ones. Further, E' and T_g of the films were high at elevated temperatures. Thus the artificial urushi films showed ideal dynamic viscoelasticity. In comparison with the film from dienylcatechol I, trienylcatechol showed higher T_g and E' at elevated temperatures. It indicates that the degree of crosslinking increases with the increase in the number of double bonds in the side chain.

The results indicate the following requirements for film formation in the alkenylcatechol derivatives. (1) The degree of methylene-interrupted double bonds in the side chain needs to be at least two. The presence of a bisallylic methylene group is known to be indispensable in the initial step of the polymerization of urushi^{12,13}. Actually no film could be obtained in the case of alkenylcatechol having the side chain with only one double bond. Thus, we conclude that the most plausible cause is the lack of such a methylene group. (2) The double bond configurations of the side chain needs to be *cis*. The *cis* double bond can be crosslinked at a greater extent than the *trans* one by the laccase from *Pycnoporus coccineus*. Since the definite specificity was dependent on the stereo structure, it may result from substrate specificity of laccase-*Pyc*. It is believed that in the polymerization process of urushi only the initial oxidation of urushiol to urushiol-quinone is catalyzed by laccase¹². Thus, it is possible that laccase-*Pyc* selectively catalyzes that oxidation of the catechol derivatives having *cis* double bonds of the side chain to the corresponding *o*-quinones. The exact cause for this phenomenon, however,

remains unsolved and is under study.

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