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Dedicated to the 80th Birthday of Dr. Otto Vogl, Herman F. Mark Professor Emeritus

Laccase-Catalyzed Curing of Natural Phenolic Lipids and Product Properties

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This paper describes the laccase-catalyzed curing of phenolic lipids from renewable resources. The phenolic lipids employed (urushiol, laccol and thitsiol) are the saps of oriental lacquer trees. The curing of urushiol and laccol efficiently proceeded in the presence of protein hydrolysate, yielding the crosslinked film with high gloss surface and high hardness. Dynamic viscoelasticity analysis and FT-IR monitoring of the curing suggested that the crosslinking mechanism is similar to that of the natural urushi. This study provides a new methodology of the enzymatic curing for coating materials from inexpensive renewable resources; therefore, the present method may provide with a future environmentally benign process of coating.

Keywords: curing; enzymatic catalysis; laccase; laccol; natural phenolic lipid; thitsiol; urushiol

1 Introduction

For a long period, oriental lacquer ("Urushi") has been used for traditional coating objects like daily life's wares and Buddha statues owing to excellent property and beauty in Japan (1). Urushi coating exhibits solvent resistance, excellent toughness and brilliance for a long time, even longer than one thousand years in some cases in comparison with synthetic coatings. Therefore, Urushi coating is regarded as one of the most typical symbolic materials in Japanese arts and culture. It is to be noted that Urushi is the only example material actually utilized, which is hardened by enzymatic catalysis in nature. However, the current production of Urushi sap has been greatly decreased in Japan and most of starting saps for manufacturing Urushi wares are imported from abroad.

Oriental lacquer from Japan, China and Korea, is a crosslinked (cured) natural resinous sap of the *Rhus vernicifera* tree (2). The oleoresins of the sap is called "urushiol", which consists of a mixture of catechol derivatives having an unsaturated hydrocarbon chain mainly with 1–3 double bonds at 3-position of catechol (Scheme 1) (3).

Over the century, Urushi has been studied as an important subject from the organic chemistry viewpoint in Japan. In particular, the chemical structure and composition of urushiol have been repeatedly studied and characterized from time to time. The first study was performed by Ishimatsu in 1878 and soon after by Yoshida (4). In the early days of 20th century, pioneering works by Majima revealed the structure of main important component in the sap, i.e.,"urushiol" (5). In the 1950s, Dawson studied structure aspects of urushiols and exactly identified them as 3-*n*-alkyl and 3-*n*-alkenylcatechols (6). Two decades later, Kumanotani investigated the components of the sap, particularly by using the modern analytical spectroscopic methods (7). The composition of the raw Urushi sap from Japan is typically in about 65% urushiol, 8% water-soluble polysaccharides, 2% glycoprotein, less than 1% laccase, and 25% water. The Urushi sap is considered as a water in oil dispersion system.

The *Anacardiaceae* is a moderately large family containing about 80 genera and about 600 species (8). The plants of a few genera in this family produce a sap that is suitable for the basis of exquisite coating materials for art objects (1). The

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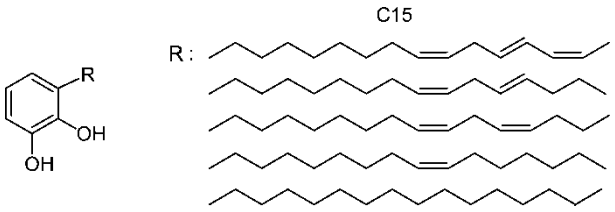


Sch. 1. Structure of Urushiol.

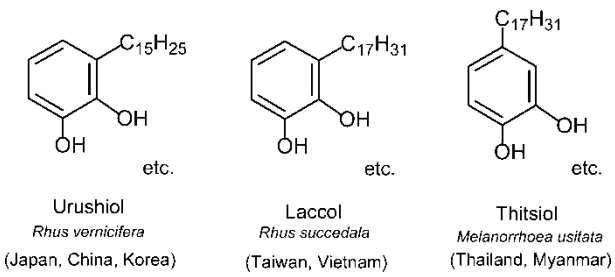
species *Rhus vernicifera* tree giving urushiol belongs to the *Anacardiaceae* family. Similar species, *Rhus succedala* and *Melanorrhoea usitata*, are found in some East Asian Countries. The sap from these trees is called "laccol" (Taiwan and Vietnam) and "thitsiol" (Thailand and Myanmar), respectively. These natural phenolic lipids are shown in Scheme 2. They have also been used as coating materials; however, these are inferior to urushiol in hardness and mechanical properties after cured, and their enzymatic curing proceeds slowly as compared with urushiol.

Crosslinking of urushiol is supposed to be accomplished via two stages; that is, by the laccase-catalyzed oxidative coupling of the urushiol phenol moiety and then by the subsequent auto-oxidation of the unsaturated hydrocarbon chain in air (1, 7). The first stage involves the oxidation of the catechol moiety of urushiol to give a semiquinone radical and/or an *o*-benzoquinone species, which is subjected to reaction by a number of competing pathways, yielding a mixture of dimer and oligomers with complicated structures.

The curing of Urushi sap proceeds under air at room temperature without organic solvents, therefore, Urushi coatings system can be regarded as an environmentally benign process, which is strongly demanded for future coating industry. However, modeling studies of Urushi have been scarcely attempted (9). This is mainly due to the difficulty in the preparation of urushiol.

Recently, we have developed the curing of new urushiol analogues to produce an "artificial Urushi" (10). The analogues were designed and conveniently synthesized by regioselective esterification of phenol derivatives having a primary alcohol with an unsaturated fatty acid using lipase catalyst. We also regarded cardanol as an urushiol analogue. Cardanol, a main component obtained by thermal treatment of cashew-nut-shell liquid (CNSL), is a phenol derivative having a C15 unsaturated hydrocarbon chain with mainly 1–3 double bonds at meta position. CNSLs were chemoselectively polymerized by soybean peroxidase or by a model metal complex catalyst to form a soluble polymer having unsaturated reactive groups. The product prepolymer from the urushiol analogue was cured by thermal treatment or by cobalt naphthenate catalyst to yield an urushi-like glossy film.

In this paper, a new enzymatic curing system was developed using renewable resources of the natural phenolic lipids as a starting substance. Moreover, the physical properties of the resulting film were examined and the curing behavior was investigated. The improvement of drying time and mechanical properties of the film are expected to be useful for application in practical use of artificial Urushi. These new results will expand the scope of the enzyme catalyst to the environmentally benign production of functional coating materials.



Sch. 2. General structures of natural phenolic lipids.

2 Experimental

2.1 Materials

Natural phenolic lipids, urushiol, laccol and thitsiol, were purchased from Satou Kiyomatu-Syoten Co. Ltd. in Japan. Laccase derived from *Pycnoporus coccineus* was purchased from Koken Co. Laccase derived from *Myceliophthora* was kindly donated by Novozymes Japan Ltd. Protein hydrolysate, which was obtained by hydrolyzing albuminoid with enzyme, was used (11). Other reagents were commercially available and were used without further purifications.

2.2 Purification of Natural Phenolic Lipids

The sap of the lacquer tree (urushiol from *Rhus vernicifera*) was poured into a large amount of acetone and the mixture was filtered. The filtrate was evaporated. The residue was dissolved in diethylether and was washed with water. The mixture was dried over $CaCl_2$ and the solvent was removed under reduced pressure to give purified urushiol. Similarly, laccol and thitsiol were purified.

2.3 Preparation of Sample Film

The protein hydrolysate was added in the amount of 10 wt% for urushiol, whose value is almost equal to the sum of polysaccharide and glycoprotein in Urushi. Laccase was added to the mixture and the mixing was continued under cool-blowing until the water content reached about 3 wt%. Enzyme activity of 75 units was used for the reaction of 0.5 g amount of urushiol. The sample film was prepared on a glass slide by using applicator for 50 μm thickness and kept standing at 30°C under air in the 80% humidity. Similarly, the sample film from laccol and thitsiol were prepared.

2.4 Measurements

Drying time was evaluated by a Coating Tester Kogyo RC type drying time recorder in the thermohygrostat. Film

hardness was measured by a Fischerscope H100VS microhardness tester with test force of 200 mN. Dynamic mechanical analysis was carried out by using an Orientec Rheovibron DDV-II-EP with frequency of 3.5 Hz at a heating rate of 2°C/min. FT-IR spectrum was recorded on a Perkin-Elmer Spectrum One.

3 Results and Discussion

3.1 Curing of Phenolic Lipids

Three natural phenolic lipids (urushiol, laccol, and thitsiol) were used for the enzymatic curing. The main component of laccol is similar to that of urushiol except that the carbon number of unsaturated hydrocarbon side chain is mainly C17 in laccol in comparison with C15 in urushiol. On the other hand, the main component of thitsiol is 4-substituted catechol (Scheme 2) (12). The content of unsaturated side chain in laccol and thitsiol is smaller than that in urushiol. Laccase belongs to an oxidoreductase having a copper-protein moiety as active site (13). In this study, laccase enzymes derived from *Pycnoporus coccineus* and *Myceliophthora* were used; they are highly active for oxidative polymerization of phenol derivatives. Particularly, *Pycnoporus coccineus* laccase catalyzed the curing of urushiol and the analogues effectively (10).

The laccase-catalyzed curing of urushiol, laccol and thitsiol was performed in the presence of protein hydrolysate under air in the 80% humidity at 30°C (Table 1). The protein hydrolysate, a third component (a mixture of polysaccharide and glycoprotein in urushiol), acted as emulsifier of oily urushiol and aqueous laccase solution. The sample film was prepared on a glass slide using an applicator for 50 μm thickness. The film formation by curing was observed for the samples of urushiol and laccol; however, the curing did not proceed without laccase. These data indicate that the curing took place by enzymatic catalysis. The curing of urushiol and laccol proceeded very fast; the drying time was 2-4.5 h. Especially, the drying time of the sample film using *Pycnoporus coccineus* laccase was faster. High values of hardness and Young's modulus were recorded for these four cases. On the other hand, the drying time of the film of thitsiol was over 24 h. This may be due to the higher laccase activity for 3-substituted catechol. A hard film was not obtained from thitsiol.

Table 1. Curing of natural phenolic lipids and product film properties after one week[a]

Phenolic lipid	Laccase	Drying Time (h)	Hardness[b] (N/mm^2)	Young's Modulus[b] (GPa)
Urushiol	M[c]	4.0	147	8.8
Urushiol	P[c]	2.0	162	6.9
Laccol	M[c]	4.5	134	9.9
Laccol	P[c]	2.0	156	10.3
Thitsiol	M[c]	>24	—	—
Thitsiol	P[c]	>24	—	—

[a]Film was prepared using laccase (75 units) as catalyst by adding protein hydrolysate (0.05 g) to phenolic lipid (0.5 g).
[b]Determined by a dynamic microhardness tester.
[c]M: *Myceliophthora* laccase, P: *Pycnoporus coccineus* laccase.

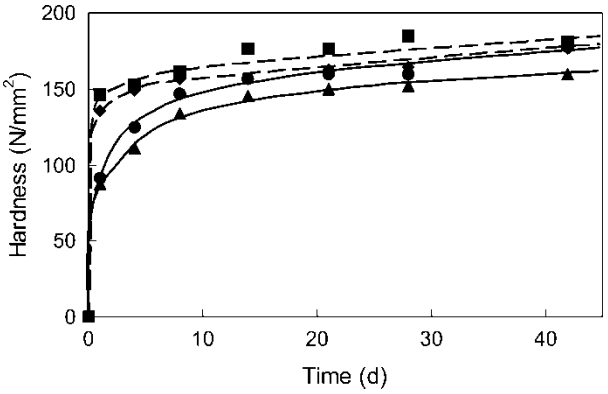


Fig. 1. Time course in hardening of the films from urushiol and laccol: (•) urushiol with *Myceliophthora* laccase; (■) urushiol with *Pycnoporus coccineus* laccase; (▲) laccol with *Myceliophthora* laccase; (◆) laccol with *Pycnoporus coccineus* laccase.

The curing of phenolic lipids was monitored by using a dynamic microhardness tester and FT-IR spectroscopy (Figure 1). At the initial stage of the curing of urushiol and laccol, the curing progressed very fast. It is normally required for the practical usage to reach the value of universal hardness of 100 N/mm^2. This value was attained within one day for urushiol as well as laccol by two laccase enzymes. Then, the universal hardness gradually increased and reached eventually the value of ca. 170 N/mm^2. The sample film of thitsiol was gradually cured; however, the value was 13 N/mm^2 after 2 months. These data indicate that a catechol having unsaturated alkyl chain at 3-position is desirable for the laccase-catalyzed curing.

Figure 2 shows FT-IR spectra of the cured film of urushiol using *Myceliophthora* laccase with a different reaction time.

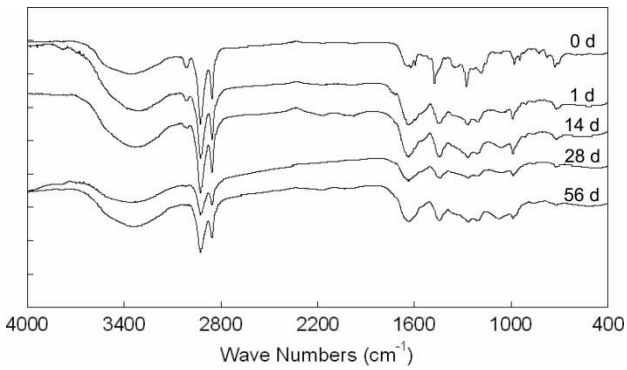


Fig. 2. Monitoring of the laccase-catalyzed curing of urushiol with *Myceliophthora* laccase using FT-IR spectroscopy.

Intensity changes of characteristic peaks using a peak at 2856 cm^{-1} due to C-H stretching vibration of the terminal methyl group as standard are shown in Figure 3. In the curing of urushiol, a peak at 3010 cm^{-1} ascribed to C-H stretching of unsaturated group in the side chain rapidly decreased in the period from 1 to 7 days (Figure 3A). This behavior corresponded well with those of the hardness (Figure 1).

The crosslinking reaction of the unsaturated moiety in the side chain was also observed by a change of characteristic C-H bending peaks at 994, 976 and 730 cm^{-1}, which are ascribed to the conjugated *cis-trans* double bond, non-conjugated *trans* double bond, and non-conjugated *cis* double bond, respectively. The intensity of the peaks at 994 cm^{-1} rapidly increased at the initial curing stage and afterwards gradually decreased. The gradual increase of the peak at 976 cm^{-1} was observed, whereas the peak intensity at 730 cm^{-1} rapidly decreased at the initial curing stage. Formation of a new peak at 1060 cm^{-1} ascribed to C-O-C linkage was also observed (Figure 3B). The peak was gradually increased, indicating the progress of the crosslinking reaction.

The broad peak at 3400 cm^{-1}, due to O-H stretching, showed a rapid decrease at the initial stage (Figure 3C). This is probably owing to the evaporation of water contained in the enzyme solution and the oxidative coupling of catechol moiety. Afterwards, the peak gradually increased, suggesting that the autoxidation of the unsaturated group in the side chain proceeds. Furthermore, the quinone was detected at the initial stage from the appearance of a new peak at 1650 cm^{-1}. These observations strongly suggest that the present curing proceeds via the fast oxidative coupling and the subsequent relatively slow autoxidation of the unsaturated group in the side chain (14).

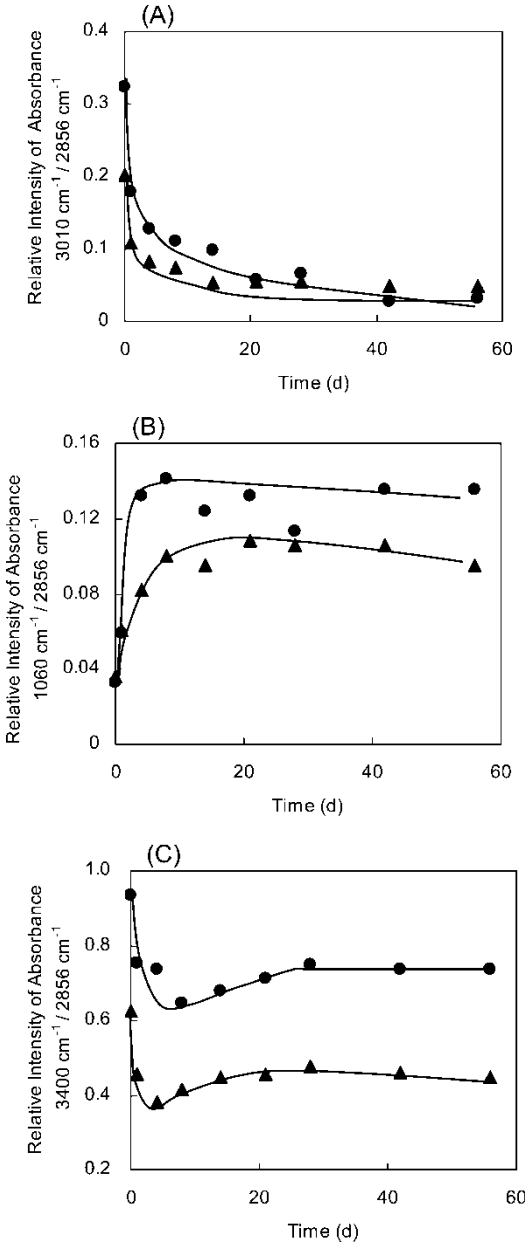


Fig. 3. Time course of relative FT-IR peak intensity in the enzymatic curing of urushiol (•) and laccol (▲) with *Myceliophthora* laccase, use of intensity at a peak of 2853 cm^{-1} as the standard: (A) 3010 cm^{-1}; (B) 1060 cm^{-1}; (C) 3400 cm^{-1}.

3.2 *Dynamic Viscoelasticity of the Cured Film*

Storage modulus (E′) and loss factor (tan δ) of the cured film after 8 weeks are shown as a function of temperature (Figure 4). With urushiol for both laccase enzymes, there was almost no different behavior. In all the samples, E′ value at room temperature exceeded 1.0×10^9 Pa and the glass transition temperature (T_g) were observed at ca. 170°C. The increase of E′ in the region of a higher temperature suggested that the unsaturated groups in the side chain remained in the measured sample. The smooth trace of tan δ means the homogeneous structure of the present cured films, suggesting that the protein hydrolysate dispersed and reoriented around the polymerized phenolic lipid (15). E′ values of the cured laccol films were lower than those of the urushiol films. These may be due to the lower crosslinking

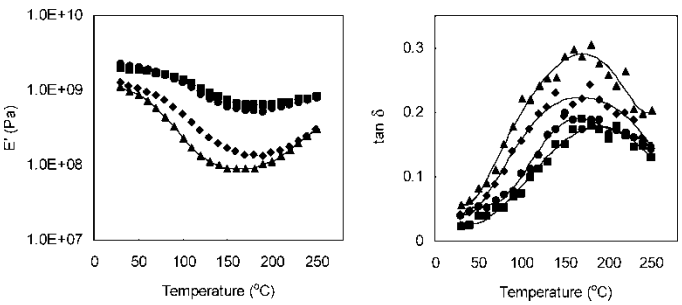


Fig. 4. Dynamic viscoelasticity of the films from urushiol and laccol: (•) urushiol with *Myceliophthora* laccase; (■) urushiol with *Pycnoporus coccineus* laccase; (▲) laccol with *Myceliophthora* laccase; (◆) laccol with *Pycnoporus coccineus* laccase.

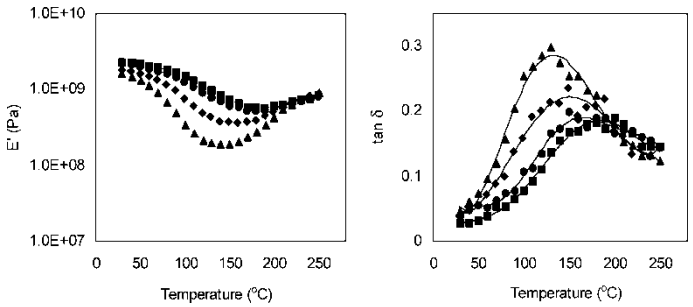


Fig. 5. Changes of dynamic viscoelasticity of urushiol with *Myceliophthora* laccase; (▲) after 2 weeks; (◆) after 4 weeks; (●) after 8 weeks; (■) after 16 weeks.

density, because laccol contains the unsaturated group in a lower amount than urushiol.

Figure 5 shows the time course of dynamic viscoelasticity of the cured urushiol film using *Myceliophthora* laccase. E′ values of the film increased with aging time and the tendency was remarkable in the high temperature region. The maximum value of tan δ decreased and T_g value shifted to a higher temperature. After 2 weeks, E′ at room temperature was 1.0×10^9 Pa and T_g was 120°C. After 16 weeks, the loss factor curve was broader and T_g value rose to 170°C. These observations are in accord with those of the above that the crosslinking reaction by enzymatic catalysis for the phenol ring is fast and that by the autoxidation of urushiol side chain is very slow. The latter took a longer time to reach a higher crosslinking density.

3.3 Effect of Laccase Amount

Table 2 shows the effects of the amount of *Myceliophthora* laccase on the cured films. With an increase of the amount, the curing time of urshiol became shorter and the universal hardness after 7 days became higher. Afterwards, the autoxidation of side chain slowly proceeded and the universal hardness of the films reached ca. 170 N/mm^2 in most cases. However, when the larger amount of 113 units of laccase for 0.5 g urushiol did not give a higher value; the amount was more than sufficient. These data suggest that the laccase amount affects the drying time, but does not affect eventual final hardness much.

Table 2. Effect of laccase amount on the curing for the film properties[a]

Laccase (units)	Drying Time (h)	Hardness[b] (N/mm^2)	Young's Modulus[b] (GPa)
19	22.0	70	10.0
38	8.5	122	6.8
56	6.0	129	6.6
75	4.0	147	8.8
94	1.5	184	9.4
113	1.5	107	6.1

[a]Film was prepared using *Myceliophthora* laccase as catalyst by adding protein hydrolysate (0.05 g) to urushiol (0.5 g). Measured after 1 week.
[b]Determined by a dynamic microhardness tester.

Similar behaviors were observed when laccol was cured. The crosslinking mechanism of the curing is considered similar to that of natural Urushi; the curing of urushiol proceeds first by the laccase catalyzed oxidative coupling of the phenol moiety and subsequently, the autoxidation of the unsaturated group in the side chain.

4 Conclusions

In this study, the laccase-catalyzed curing system was developed using natural phenolic lipids. Urushiol and laccol, having an unsaturated hydrocarbon chain at 3-position of catechol, were effectively cured in the presence of commercially available laccase enzymes to produce the crosslinked polymeric films with a high gloss surface and high hardness properties. On the other hand, thitsiol having an unsaturated hydrocarbon chain at 4-position of catechol, was slowly cured with the same enzymes and the hardness attained was low. The present curing system proceeded under mild conditions in air without organic solvent as a water in oil dispersion. Therefore, the present method is able to expand the application field of the Urushi technique and involves a great potential for a future environmentally-benign process of polymer coating, giving an example system of *green polymer chemistry* (16).

5 Acknowledgement

We wish to celebrate the 80th birthday of Professor Otto Vogl. His contribution to the polymer community worldwide is greatly acknowledged. He likes Japan, visited many times, and performed fruitful collaborations with Japanese scientists. Accordingly, he has many good friends and coworkers. He has deep knowledge in many subjects in Japan. One of such for him is Urushi as a part of Japanese culture, which is reflected by his scientific papers referred here.

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