Analysis of Fresh Sap Collected from Ryukyu Lacquer Tree

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The chemical structure of fresh lacquer sap collected from a lacquer tree growing in Nago City of Okinawa, Japan, was analyzed by gas chromatography/mass-spectrometry (GC/MS) and nuclear magnetic resonance (NMR). The results showed that Nago lacquer is laccase lacquer and its major components are 3-(heptadeca-10Z,13E,15E-trienyl)catechol, 3-(heptadeca-10Z,13E-dienyl)catechol, 3-(heptadeca-14Z-enyl)catechol, and 3-(heptadeca-12Z-enyl)catechol, which are similar to the components of Vietnamese lacquer. It showed higher laccase activity at pH 5 – 8 and better low temperature adaptability than Vietnamese lacquer. The Nago lacquer reached a dust free dry (DF) condition after 6 h, but Vietnamese lacquer did not. However, both were able to achieve harden dry (HD) in 24 h at 25°C, 80% relative humidity. In order to identify the lacquer provenance, the strontium isotope ratio was analyzed. The strontium isotope ratio (87Sr/86Sr) of Nago lacquer was 0.7110, which is different from the 0.7450 of Vietnamese lacquer.  

Keywords Chemical structure, laccase, laccase, GC/MS, NMR  
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Introduction  
Oriental lacquer sap collected from lacquer trees such as Toxicodendron vernicifluum, Toxicodendron succedanea, and Gluta usitata makes a natural coating material with a beautiful surface and high durability due to crosslinking of phenolic lipid components (urushiol, laccal, and thitsiol).1–4 These lacquer trees grow wild, but have been cultivated in several Asian countries and used as a coating material for containers, tableware, and furniture for thousands of years in China, Japan, Vietnam, Thailand, and Myanmar. Lacquer sap is a water-in-oil (W/O) emulsion and consists of lipid components that are acetone-soluble and -insoluble (acetone powder). The lipid (W/O) emulsion and consists of lipid components that are acetone powder acts as an emulsifier and a dispersing agent. These lacquer trees grow wild, but have been cultivated in several Asian countries and used as a coating material for containers, tableware, and furniture for thousands of years in China, Japan, Vietnam, Thailand, and Myanmar. Lacquer sap is a water-in-oil (W/O) emulsion and consists of lipid components that are acetone-soluble and -insoluble (acetone powder). The lipid components are the main constituents of lacquer film, and the acetone powder acts as an emulsifier and a dispersing agent. Lacquer sap dries via the laccase-catalyzed polymerization of phenolic/catecholic moieties in lipids and auto-oxidation (crosslinking) of unsaturated side chains without evaporation of an organic solvent, unlike common solvent-borne paints. Therefore, natural lacquer is an eco-friendly coating material.5–9  
  
Laccol lacquer collected from T. succedanea lacquer trees grown mainly in Vietnam and Taiwan has many chemical and physical properties similar to urushiol lacquer collected from the T. vernicifluum lacquer tree.10,11 The structure and constituents of laccol present in commercial Vietnamese lacquer sap were identified as a mixture of 3-heptadecenylcatechols containing 0 – 3 double bonds and 3-heptadecenylphenol.12  
  
Recently, Ryukyu lacquerwares have been analyzed13–15 and urushiol,16 laccal,17 and thitsiol18 were detected in these lacquerwares. However, there is no record of production of lacquer sap in Ryukyu. Where did the laccal used in Ryukyu lacquerwares come from? Whether it was imported from Vietnam or is a local material is an interesting question. In order to answer this and further future research, in this study, fresh sap collected from T. succedanea lacquer trees grown in Nago City of Ryukyu was analyzed and compared with fresh Vietnamese lacquer sap. The chemical structures, laccase activity, and drying time were examined. To identify the lacquer provenance, strontium isotopes in the lacquer sap were analyzed. The results showed that the lacquer sap collected from Ryukyu has mixed characteristics: the chemical structure is like Vietnamese lacquer laccal, but the properties of laccase activity, hardened dry and strontium isotope are different. Based on these results, the coating materials and techniques of ancient Ryukyu lacquerwares are also discussed.

Experimental

Materials  
Ryukyu lacquer saps were collected from one lacquer tree growing in Nago City, Okinawa Prefecture, in July, August, and September, 2014 and named Nago-7, Nago-8, and Nago-9. The sap from Toxicodendron succedanea species was kindly offered by Director Ayano Shinohara and Researcher Atsushi Abe of Okinawa Kurashima Research Center, Nago-shi, Okinawa.
Prefecture. A fresh Vietnamese lacquer collected from a *T. succedanea* lacquer tree growing in Tam Nong District, Phu Tho Province, Vietnam, in 2014 also was analyzed as a comparative sample. The saps from *T. succedanea* species were kindly offered by Prof. Bach Phuc, Research Centre for Polymer Materials, Hanoi University of Science and Technology.

Because June to October is a good season to collect lacquer sap and July to September the best season, we selected three samples collected in July, August, and September to compare with Vietnamese lacquer.

All lacquer saps were filtered through filter paper before being analyzed. Syringaldazine was purchased from Aldrich (St. Louis, MO). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, a silylating agent) was purchased from TCI Company (Tokyo, Japan). Other chemicals were reagent grade and all reagents were used directly without further purification.

**Laccase activity**

The laccase activity contained in lacquer sap was determined by ultraviolet-visible (UV-Vis) spectrometry using syringaldazine. UV-Vis spectra were recorded using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a temperature-controllable cuvette holder TCC-240A (Shimadzu). Crude enzyme solutions that contain laccase and oligopolysaccharides were separated from 10 mg of acetone powder using 10 mL of cold deionized water, and the acetone powder was separated from raw lacquer sap and dried under vacuum without heat to avoid inactivation of enzyme. The laccase activity was measured at 30°C with pH 6.5 buffer and one unit was defined as a δAbs at 530 nm of 0.001 per min in a 3-mL reaction mixture. The volume of enzyme used was 0.5 mL. In order to confirm the laccase activity, pure laccase from *T. vernicifluum* purchased from Sigma Chemical was used as the standard.

**Evaluation of drying properties**

The drying properties of lacquer saps were examined using a drying time recorder (Tayu Equipment, Osaka, Japan) according to the JIS-K-5400 (Japanese Industry Standard, Kagaku) testing method.

**87Sr/86Sr isotope ratio**

The 87Sr/86Sr isotope ratio was analyzed according to our previous report.

**GC/MS analysis**

Gas chromatography/mass-spectrometry (GC/MS) was carried out using an Agilent 6890N/5975 GC/MS system (Agilent Technologies, Santa Clara, CA). Samples were silylated to improve the S/N ratio before introduction by a GC injector: ca. 2.2 eq. of BSTFA was added to 2.0 mg/mL of the sample solution (solvent: acetone). GC conditions were injection at 280°C, split ratio of 50:1, and the carrier gas was He (1.0 mL/min). An Agilent DB17 GC column (0.25 i.d. × 0.25 mm × 30 m) was used and column temperature was set at 250°C.

**NMR analysis**

Nuclear magnetic resonance (NMR) spectra were recorded by a JNM-ECA500 (JEOL, Tokyo, Japan, 1H: 500.16 MHz, 13C: 125.77 MHz) using quartz sample tubes (diameter: 5 mm). Chloroform-d (CDCl3) and dimethylsulfoxide (DMSO-d6) were used as deuterated solvents, and tetramethylsilane (TMS) as the internal standard. Lacquer samples (10 – 40 mg) were dissolved in 0.5 mL deuterated solvents, and then one drop of deuterium oxide (D2O) was added to NMR sample tubes containing CDCl3 in order to eliminate signals attributed to hydroxyl groups and unnecessary H2O.

**Results and Discussion**

**Laccase activities**

Syringaldazine was used as a substrate to determine the
relationship between laccase activity and pH, activity and temperature by ultraviolet-visible spectrometry.20 The results are shown in Fig. 1.

It can be observed from Fig. 1A that at the optimum pH 5.0 – 8.0, Nago-7 and Vietnamese lacquer showed almost the same activity at about 70 units, Nago-8 and Nago-9 showed about 100 and 110 units, higher than Nago-7 and Vietnamese lacquers. This can be considered due to the collection season. In Ryukyu, August and September are the typhoon season; the average monthly rainfalls are 240.5 and 260.3 mm, respectively. On the other hand, the rainfall of July averages about 141.4 mm.21 In general, relatively higher rainfall and temperatures are more suitable for plant physiological activity.

From Fig. 1B, it can be concluded that the optimum temperature for Nago laccases is about 55°C and that for Vietnamese lacase is about 65°C, but the reason is not clear. Although both Nago and Vietnamese lacquer trees belong to the T. succedanea species, due to the different growth environment, the number and types of trace elements contained in laccase are a little different due to the optimum temperature difference.

Drying properties

The drying times of Nago and Vietnamese lacquers are summarized in Table 1. Nago lacquers reached the dust free dry (DF) state after 6 h, touch free dry (TF) after 12 h, and complete hardness dry (HD) after 24 h. In comparison, Vietnamese lacquer was still not dry (ND) after 6 h, but was the same TF dryness as Nago lacquers after 12 h.

87Sr/86Sr isotope ratio of laccol

Because the concentration of strontium isotopes contained in agricultural products is related to the soil in which they are grown,22 it is used to differentiate lacquer saps collected from different lacquer trees grown in different countries and areas. In general, lacquer sap from Chinese mainland and Japanese islands show different 87Sr/86Sr isotope ratios due to the different geological ages of soil. The Sr isotope ratio in lacquer sap was found to be the same as that of the soil in which a lacquer tree was grown, and this character can be used to determine the provenance of lacquer from trees of the same species but grown on different continents, such as the Asian continent and Japanese islands. From the results of this study, it is possible to identify Ryukyu and Vietnamese lacquers of the same T. succedanea tree species. The strontium isotope ratios (87Sr/86Sr) of Nago-7 and Vietnamese lacquer saps were analyzed according to our previous report19. 87Sr/86Sr = 0.7110 of Nago-7 and 87Sr/86Sr = 0.7450 of Vietnamese lacquers were obtained. The different values can be considered due to the different environments in which the lacquer trees were grown.

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Table 1  Drying time of Nago and Vietnamese lacquer saps

| Sample      | 2   | 4   | 6   | 12  | 24  |
|-------------|-----|-----|-----|-----|-----|
| Nago-7      | ND  | ND  | DF  | TF  | HD  |
| Nago-8      | ND  | ND  | DF  | TF  | HD  |
| Nago-9      | ND  | ND  | DF  | TF  | HD  |
| Vietnam     | ND  | ND  | ND  | TF  | HD  |

a. Wet thickness: 76 μm, drying conditions: 25°C, 80%RH, ND: none dry, DF: dust free dry, TF: touch free dry, HD: harden dry.
Table 2 Characteristics of Nago and Vietnamese lacquer saps by GC/MS

| Peak No. | Mw [M]+ | Area of lipid monomer, %a | Side chain structure |
|----------|---------|---------------------------|---------------------|
| Nago-7   | 464     | 3.4 4.7 5.2 1.3            | -C17H31             |
| Nago-8   | 462     | 0.2 0.3 0.4 0.3            | -CH3(CH=CH-C6H5)    |
| Nago-9   | 462     | 0.4 0.3 0.4 0.3            | -CH3(CH=CH-C6H5)    |
| Vietnam  |         |                           |                     |
| 1        | 492     | 6.7 5.4 3.7 2.9            | -C17H31             |
| 2        | 490     | 5.3 4 6.1 2               | -CH3(CH=CH-C6H5)    |
| 3        | 490     | 18.1 23.9 28 23.8         | -C17H31             |
| 4        | 490     | 20.7 19.9 20.8 9.4        | -C17H31             |
| 5        | 490     | 12.9 10.4 10.1 15.9       | -C17H31             |
| 6        | 490     | 2.5 3.3 3 3.8             | -C17H31             |
| 7        | 486     | 1.6 2 2.2 2.1             | -C17H31             |
| 8        | 488     | 6.8 6 6.5 4.7             | -C17H31             |
| 9        | 488     | 20.6 18.8 13 30.7         | -C17H31             |
| 10       | 486     | 0.9 1 0.7 2.8             | 3-(Heptadecatrienyl)-catechol |

a. Determined by GC/MS using silylation method.

Chemical structures of laccol

The chemical structures and proportions of laccol monomer extracted by acetone from Nago-7, Nago-8, Nago-9, and Vietnamese lacquer saps were identified by GC/MS analysis using the silylation method. The total ion chromatograms (TIC) are shown in Fig. 2.

All four TICs showed 13 peaks total, which were assigned according to each mass chromatogram. The results are summarized in Table 2.

The main peak 6 was determined to be silylated 3-(heptadeca-12Z-enyl)catechol, peak 7 was silylated 3-(heptadeca-14Z-enyl)catechol, and peak 12 was silylated 3-(heptadeca-10Z,13E-trienyl)catechol, respectively. Vietnamese lacquer showed higher peak 12 and lower peak 7 compared with Nago lacquers.

Figure 3 is the proton NMR of laccol monomer separated from Nago-7 and Vietnam lacquer saps. At 0.5 – 3.0 ppm, the two NMR spectra show almost the same profile, and at 4.5 – 7.0 ppm, the two NMR spectra also show almost the same profile; the small difference in the peak area suggests that Nago and Vietnamese laccol have almost the same chemical structure. We also analyzed Nago-7 in detail by 1H-NMR, 13C-NMR, and two-dimensional NMR including double quantum filtered-correlation spectroscopy (DQF-COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC), and IR measurements, and the results were almost completely consistent with the GC/MS results.

From the above analysis, the laccol separated from Nago and Vietnamese lacquer saps had C17-aliphatic side chains containing 0 – 3 double bonds (peaks 5 – 13). There was a small amount of phenolic lipids having C15-aliphatic side chains (peaks 1 – 3). The major components of the laccols were determined to be 3-(heptadeca-10Z,13E,15E-trienyl)catechol (peak 12), 3-(heptadeca-10Z,13E-dienyl)catechol (peak 8), 3-(heptadeca-14Z-enyl)catechol (peak 7), and 3-(heptadeca-12Z-enyl)catechol (peak 6), respectively.

Conclusions

In this study, the chemical structures and the proportions of laccol separated from lacquer trees grown in Nago of Ryukyu and Vietnamese lacquers were analyzed by GC/MS and NMR measurements. These laccols were identified as 3-(heptadeca-10Z,13E,15E-trienyl)catechol, 3-(heptadeca-10Z,13E-dienyl)catechol, 3-(heptadeca-14Z-enyl)catechol, and 3-(heptadeca-12Z-enyl)catechol. Although the chemical structure of Nago lacquer has the characteristic of laccol, the properties are different: the maximum laccase activity occurs at a different pH, the drying time is different, and the 87Sr/86Sr isotope ratio is different due to different growth environment. The same laccol chemical structure but different 87Sr/86Sr isotope ratio will guide the future conservation and restoration of ancient Ryukyu lacquerwares. Based on this result, further analysis of ancient Ryukyu lacquerwares should reveal the source of the laccol used in ancient Ryukyu lacquerwares.

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