Effect of pH on the Dehydrogenative Polymerization of Monolignols by Laccases from *Trametes versicolor* and *Rhus vernicifera*

Takao Kishimoto,* Ayumi Hiyama, Hiroshi Toda, and Daisuke Urabe

**ABSTRACT:** Dehydrogenative polymerization of coniferyl alcohol (CA) and sinapyl alcohol (SA) was conducted using commercial laccases, fungal laccase from *Trametes versicolor* (*LacT*) and plant laccase from *Rhus vernicifera* (*LacR*), at pH 4–7 to investigate how the enzymatic polymerization of monolignols differs between these two laccase systems. The enzyme activity of *LacT* was the highest at pH 4, whereas that of *LacR* was the highest at pH 7. A dehydrogenation polymer (DHP) was obtained only from CA in both laccase systems, although the consumption rate of SA was higher than that of CA. 1H-13C HSQC NMR analysis showed that DHPs obtained using *LacT* and *LacR* contained lignin substructures, including β-O-4, β-4-α-O-4, β-β, and β-5 structures. At pH 4.5, the β-O-4 structure was preferentially formed over the β-O-4/α-O-4 structure, whereas at pH 6.5, the β-O-4/α-O-4 structure was preferred. The pH of the reaction solution was more vital to affect the chemical structure of DHP than the origin of laccases.

**INTRODUCTION**

Lignin is one of the most abundant biopolymers found in nature. The lignin structure is quite complicated and comprises many substructures, including β-O-4, β-S, β-β, S-S, and O-S structures as shown in Figure 1A.1 These substructures are formed from the enzymatic dehydrogenative polymerization of monolignols, including coniferyl alcohol (CA), sinapyl alcohol (SA), and p-coumaryl alcohol (Figure 1B). Nontraditional monolignols are also reported to be involved in lignin biosynthesis.2 These monolignols are oxidized by peroxidases and/or laccases, and the resulting monolignol radicals couple with each other to produce lignin dimers. Under endwise polymerization, the dimers are further oxidized and coupled with monolignol radicals to form lignin trimers. Further oxidation of the phenolic end group of the growing polymer and coupling with monolignol radicals are repeated to produce lignin.3-5

To investigate the structure of natural lignin, synthetic lignin derived from the dehydrogenative polymerization of monolignols has long been used.7 In previous investigations, dehydrogenative polymerization of CA and SA was conducted using horseradish peroxidase (HRP), and the presence of both guaiacyl and syringyl units increased the β-O-4 structures in natural and synthetic lignins.8 Silver(I) oxide was also used to investigate the radical coupling of monolignols. Monolignol acylates, including coniferyl acetate, sinapyl acetate, and sinapyl p-coumarylate were used, and the effects of acyl groups on the lignin substructures were studied.6,7

Recent investigations on lignin biosynthesis suggest that peroxidase/H2O2 and laccase/O2 play an essential role in lignin polymerization in many plant species.8,9 Especially, laccase draws increasing attention, and the vital role of laccases in the lignification was shown through the knockout mutants in *Arabidopsis*.10-12 However, studies on synthetic lignins obtained from the dehydrogenative polymerization of monolignols using laccases have drawn less attention. Only a few studies were reported on the structural analysis of synthetic lignins obtained using laccases.13-16

Laccase is a multicopper oxidase with a high degree of structural conservation among bacteria, fungi, and plants.17 Basic enzyme activities of laccases are well elucidated using various substrates, including hydroquinone, guaiacol, catechol, caffeic acid, 2,6-dimethoxyphenol, and syringaldazine.17,18 The optimal pH of laccase activity for the phenolic substrate depends on the origin of laccases.17 The optimal pH for phenolic substrates is 3–5 for most fungal laccases, including *Trametes versicolor*. For plant laccases, the optimal pH range is 5–7.

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In this study, the dehydrogenative polymerization of CA and SA was performed using commercial laccases, fungal laccase from *T. versicolor* (*LacT*) and plant laccase from *Rhus vernicifera* (*LacR*), to study how the dehydrogenation polymerization varies between these laccase systems. The enzyme activities of laccases were compared at different pHs. In particular, the effect of pH on the structure of the obtained dehydrogenation polymer (DHP) was analyzed using $^{1}H-^{13}C$ correlation heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy.

**MATERIALS AND METHODS**

**General.** All chemicals were bought from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) or Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). CA and SA were synthesized from ethyl ferulate and ethyl sinapate, respectively. NMR spectra were recorded using a Bruker AVANCE Neo 500 FT-NMR (500 MHz) spectrometer or a Bruker Japan (Tokyo, Japan). The molecular mass of CA, SA, and 3,4-dimethoxy phenol were 12.4, 13.3, and 36.5 kDa, respectively, using sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The enzyme activities of laccases were determined using 2,6-dimethoxyphenol as a substrate. A 2,6-dimethoxyphenol solution (0.1 mol/L, 500 μL), a buffer solution (50 mmol/L, pH 4–7, 500 μL), and an aqueous laccase solution (*LacT*: 0.05 mg/mL, 500 μL; *LacR*: 0.25 mg/mL, 500 μL) were mixed, and the increase in absorbance was measured at 470 nm at 30 °C for 10 min using a spectrophotometer (Shimazu UV-1800, Kyoto, Japan). One unit of the enzyme was defined as the amount that produced 1 μmol of the oxidized product (coerulignone, 49.6 mM$^{-1}$ cm$^{-1}$ at 470 nm) in 1 min.

**Dehydrogenative Polymerization of Monolignol by Laccase.** In a typical procedure, to a stirred solution of CA (18.0 mg, 0.1 mmol) or SA (21.0 mg, 0.1 mmol) in 9 mL of a buffer solution (pH 4–7) in a round bottom flask (25 mL), 1 mL of laccase solution (0.5 mg/mL for *LacT* or 3 mg/mL for *LacR*) was added at 30 °C (“Zulauf” method). At a prescribed time, an aliquot (250 μL) of the reaction mixture was withdrawn, and ethyl acetate (500 μL), aq 3,4-dimethoxycacetophenone solution (1.8 mg/mL, 250 μL, internal standard), and 0.1 mol/L HCl solution (250 μL) were added to the mixture. The mixture was shaken, and ethyl acetate solution was dried over Na$_2$SO$_4$ and concentrated to dryness in vacuo. The reaction products were diluted using methanol (500 μL) and analyzed using high-performance liquid chromatography (HPLC) on a Gilson liquid chromatography system (WI, USA) with a UV/vis detector model 118 (2022, 7, 9852). A YMC-Triart Phenyl column (15 cm $\times$ 4.6 mm) (Kyoto, Japan) was used at 30 °C. The solvent system was a gradient of methanol (A) and 0.1% formic acid (B) with a flow rate of 1.0 mL/min. The gradient was as follows: 25% A for 30 min, and from 25 to 50% A in 25 min. The retention times of CA, SA, and 3,4-dimethoxy phenol were 12.4, 13.3, and 36.5 min, respectively.

![Chemical structures. (A) Substructures in softwood lignin. The most abundant β-O-4 structure is highlighted in red. (B) Monolignols.](https://doi.org/10.1021/acsomega.2c00144)
After the completion of the reaction, the reaction mixtures were centrifuged at 3000 rpm (approx. 1500 g) for 15 min. The precipitates were washed using water, lyophilized, and further dried in vacuo over P₂O₅ to give DHP. The supernatant solution was extracted using ethyl acetate (10 mL × 3). The ethyl acetate solution was washed with brine, dried over Na₂SO₄, and concentrated to dryness in vacuo to give ethyl acetate extracts that were unprecipitated.

**Size Exclusion Chromatography of Dehydrogenative Polymerization Products.** The obtained DHP was acetylated with acetic anhydride and pyridine and analyzed using size exclusion chromatography. The sample solution was filtered and injected into Shodex GPC packed columns GPC KF-802 + KF-803L × 2 (30 cm × 8 mm) using a JASCO liquid chromatography system equipped with a UV/vis detector UV-975 (Tokyo, Japan). Tetrahydrofuran was used as an eluent with a flow rate of 1.0 mL/min at 40 °C. Polystyrene standards PSTQuick E and F (Tosoh, Tokyo, Japan) were used for molecular mass calibration.

**RESULTS AND DISCUSSION**

**Enzyme Activities of Laccases from** *T. versicolor* and *R. vernicifera at Different pHs.** The enzyme activities of LacT and LacR were measured in sodium acetate buffer (pH 4–4.5) and potassium phosphate buffer (pH 4–7) using 2,6-dimethoxyphenol as a substrate. LacT was used without purification. LacR was purified before use. The protein contents in LacT and purified LacR were 17.9 and 27.7%, respectively. The relative activity of LacT is indicated in Figure 2A. The maximum enzyme activity of LacT was 156 unit/g (100%) at pH 4.0 in acetate buffer. In a phosphate buffer, the activity was a little lower than that in acetate buffer. The enzyme activity of LacT for the oxidation of 2,6-dimethoxyphenol reduced with the increase in the pH, and it was almost lost at pH 7.0 in the phosphate buffer. Most fungal laccases have an optimum pH range of 3.0–5.0 for phenolic substrates.

In contrast, the enzyme activity of LacR was the highest at pH 7.0 (10.7 unit/g, 100%) in the phosphate buffer, as shown in Figure 2B. The activity reduced with a reduction in pH, and it was only 1.1 unit/g at pH 4.0 in the phosphate buffer. In the acetate buffer, the enzyme activity of LacR was significantly higher than that in the phosphate buffer. The high enzyme activity under neutral conditions for LacR using 2,6-dimethoxyphenol as a substrate was consistent with the reported observation using isoeugenol and CA as substrates. Plant laccases have their optimum pH range nearer to the physiological range. The enzyme activity of LacR was much lower than that of LacT, and the optimum pH range differed from each other. The basic enzyme activities of LacR and LacT were quite different from each other.

**Dehydrogenative Polymerization of CA and SA by Laccases from** *T. versicolor* and *R. vernicifera.* The phosphate buffer was used for the dehydrogenative polymerization of monolignols because acetic acid was introduced into the collected DHP molecules in preliminary experiments when acetate buffer was used. Monolignols, CA, and SA were treated separately with LacT and LacR at pH 4.5 and 6.5 in phosphate buffer. An aliquot of the reaction mixture was withdrawn at a prescribed time, and residual monolignols were analyzed by HPLC. The reactivity of monolignols using LacT is indicated in Figure 3A. The enzyme activity of LacT for CA and SA at pH 4.5 was higher than that at pH 6.5, which was the same as that when 2,6-dimethoxyphenol was used as a substrate. For CA and SA, almost all monolignols were consumed within 4–5 h at pH 4.5, whereas at pH 6.5, more than 60% of monolignols remained unreacted after 6 h reactions. The reactivity of SA toward LacT was slightly higher than that of CA at pH 4.5 and 6.5.

**Figure 2.** Effect of pH on the enzyme activity using 2,6-dimethoxyphenol as a substrate. (A) Relative activity of LacT. Solid circle: acetate buffer, solid triangle: phosphate buffer. The maximum activity was 156 unit/g (100%) at pH 4 in sodium acetate buffer. (B) Relative activity of LacR. Open circle: acetate buffer and open triangle: phosphate buffer. The maximum activity was 10.7 unit/g (100%) at pH 7 in potassium phosphate buffer.

**Figure 3B** shows the reactivity of monolignols by LacR at pH 4.5 and 6.5. The enzyme activity of LacR for CA and SA at pH 6.5 was higher than that at pH 4.5. The difference in the reactivity of monolignols between pH 4.5 and pH 6.5 was more evident when SA was used as a substrate. SA was completely consumed by LacR at pH 6.5 in 4–6 h, whereas CA needed more than 10 h to complete the reactions at pH 4.5 and pH 6.5. The higher reactivity of SA than CA was observed both at pH 4.5 and pH 6.5, which was the same as when LacT was used. The higher enzyme activity for SA than CA was reported for maple laccase and *Rhus* laccase at neutral pH.

**Table 1** summarizes the yields of DHP and ethyl acetate extracts from the dehydrogenative polymerization of CA and SA by LacT and LacR. After monolignols were completely consumed, reaction mixtures were centrifuged, and precipitates were obtained and dried. The obtained precipitate was referred to as DHP. The yield of DHP from dehydrogenative polymerization of CA by LacT at pH 4.5 was 68 wt %, which was higher than that at pH 6.5. In contrast, SA did not form DHP by LacT at any pH, although the consumption of SA by LacT was faster than that of CA. The reaction products from SA by LacT were recovered as ethyl acetate extracts.
dimer compound was not considered DHP. These results indicate that LacR cannot produce DHP from SA at neutral or acidic pHs. It was also reported that DHP from SA was hardly produced by Rhus lacca at pH 6.5 by the Zutropf method (gradual addition of monolignol: end-wise polymerization).16 From our experimental data and the reported results, it can be concluded that either fungal laccase LacT or plant laccase LacR cannot produce DHP from SA at acidic or neutral pHs.

**Structural Analysis of DHP from CA by Laccases from T. versicolor and R. vernicifera.** Table 2 shows the molecular mass of the DHPs obtained from dehydrogenative polymerization (Zulauf method: bulk polymerization) of CA by LacT at pH 4.5 and 6.5, and using LacR at pH 6.5. Weight average molecular mass ($M_w$) of the DHP from CA by LacT at pH 4.5 was 1197, corresponding to a tetramer or pentamer. $M_w$ of DHPs from CA by LacT at pH 6.5 and by LacR at pH 6.5 was similar and corresponds to a trimer. Polydispersities of DHPs were small, and they are identical to each other ($M_w/M_n = 1.2-1.3$).

| entry | laccase | pH  | $M_w$ (±) | $M_n$ (±) | $M_w/M_n$ |
|-------|---------|-----|-----------|-----------|-----------|
| 1     | LacT    | 4.5 | 914 ± 55  | 1197 ± 90 | 1.31 ± 0  |
| 2     | LacT    | 6.5 | 715 ± 33  | 867 ± 43  | 1.21 ± 0  |
| 3     | LacR    | 6.5 | 679       | 853       | 1.26      |

The molecular mass of the DHP obtained from CA by LacT and LacR in this investigation was small compared with those obtained using HRP in our previous investigations.5 These experiments using LacT and LacR were conducted using the Zulauf method (addition of monolignol at once), whereas the previous HRP experiments were conducted using the Zutropf method. The gradual addition of monolignol (Zutropf method) leads to the end-wise polymerization, which makes the molecular mass of DHP higher. The low molecular mass of the DHP in this investigation is partly due to the Zulauf method. Further experiments are necessary to clarify the effect of laccase on the molecular mass of DHP.

Figure 4 shows the HSQC NMR spectra of DHPs from dehydrogenative polymerization of CA using LacT and LacR in DMSO-$d_6$. HSQC NMR analysis can provide detailed information on the lignin substructures in the DHP obtained using LacT and LacR. Lignin substructures $\beta$-$O$-$4$ (A), $\beta$-$S$ (B), and $\beta$-$\beta$ (C) were seen in all DHP samples (Figure 4). The basic structure of DHPs obtained using LacT and LacR was similar to those obtained by enzymatic polymerization of CA using HRP. In addition to the primary lignin substructures, a minor lignin substructure, $\beta$-$O$-$4/\alpha$-$O$-$4$ (A'), was observed. The $\beta$-$O$-$4/\alpha$-$O$-$4$ (A') structure has not been reported in softwood or hardwood lignins.20,21 The $\beta$-$O$-$4/\alpha$-$O$-$4$ (A') structure was undetected in milled wood lignin isolated either from todic or white birch in our investigations.22,23 In contrast, the $\beta$-$O$-$4/\alpha$-$O$-$4$ (A') structure was observed in mature and immature bamboo lignins in our previous investigations.24,25 Bamboo-cultured cell lignin also contains the $\beta$-$O$-$4/\alpha$-$O$-$4$ (A') structure.26

Table 3 shows the frequency of the lignin substructures in DHPs, which was estimated by regarding $\beta$-$O$-$4$ (A), $\beta$-$O$-$4/\alpha$-$O$-$4$ (A'), $\beta$-$S$ (B), and $\beta$-$\beta$ (C) structures totally as 100% of the side chain structures. The signal intensities of $\beta$-$O$-$4$ (Aa), $\beta$-$O$-$4/\alpha$-$O$-$4$ (A'a), $\beta$-$S$ (Ba), and $\beta$-$\beta$ (Ca) were used for the

After the 10 h reaction of CA by LacR at pH 6.5, 60 wt % of DHP was collected, whereas only a trace amount of DHP was derived from CA by LacR at pH 4.5. Alternatively, SA did not form DHP at all using LacR at pH 4.5. When SA was treated using LacR at pH 6.5, a significant amount of precipitate (25 wt %) was obtained. However, the precipitate was found to be a $\beta$-$\beta$ type dimer (syringaresinol) by NMR analysis. Thus, this
analysis. For LacR at pH 4.5, ethyl acetate extracts were assessed instead of DHP because only a trace amount of DHP was obtained under the conditions used.

The most abundant substructure in DHP obtained using LacT at pH 4.5 (Zularf method) was the \(\beta\)-S (B) structure (56.8%), followed by the \(\beta\)-\(\beta\) (C) (30.5%) and the \(\beta\)-O-4 (A) structures (11.7%). A small amount of the \(\beta\)-O-4/\(\alpha\)-O-4 (A') structure (1.1%) was also observed at pH 4.5. Similarly, in ethyl acetate extracts collected using LacR at pH 4.5, the frequency of the \(\beta\)-S (B) structure (59.3%) was the highest.

Table 3. Relative Abundance of Substructures in DHPs Estimated by HSQC NMR Analysis

| entry | laccase | pH  | \(\beta\)-O-4   | \(\beta\)-O-4/\(\alpha\)-O-4 | \(\beta\)-S   | \(\beta\)-\(\beta\) |
|-------|---------|-----|----------------|---------------------------|-------------|----------------|
| 1     | LacT    | 4.5 | 11.7 ± 0       | 1.1 ± 0.2                 | 56.8 ± 1.0 | 30.5 ± 1.2   |
| 2\(^a\) | LacR   | 4.5 | 20.1 ± 0.2     | 0.2 ± 0.2                 | 59.3 ± 0.1 | 20.5 ± 0.5 |
| 3     | LacT    | 6.5 | 3.4 ± 0.3      | 18.8 ± 0.8                | 47.0 ± 0.6 | 30.8 ± 0.1 |
| 4     | LacR    | 6.5 | 1.9 ± 0.2      | 26.4 ± 0.4                | 44.6 ± 1.6 | 27.1 ± 1.0 |

\(^a\)Ethyl acetate extracts were analyzed because DHP (precipitates) was not obtained.

Figure 4. HSQC NMR spectra of DHPs from dehydrogenative polymerization of CA by (A) LacT at pH4.5, (B) LacT at pH6.5, and (C) LacR at pH6.5 in DMSO-d<sub>6</sub>.
followed by the $\beta\beta$ (C) (20.5%) and the $\beta$-$O$-$4$ (A) structures (20.1%). The frequency of the $\beta$-$O$-$4$/\alpha$-$O$-$4$ (A′) structure (0.2%) was low.

Alternatively, in the case of LacT at pH 6.5, the proportion of the $\beta$-$5$ (B) was the highest (47.0%), followed by the $\beta\beta$ (C) (30.8%) structures. The amount of the $\beta$-$O$-$4$/\alpha$-$O$-$4$ (A′) structure became more significant, and the relative proportion of the $\beta$-$O$-$4$/\alpha$-$O$-$4$ (A′) structure reached 18.8%. The frequency of the $\beta$-$O$-$4$ (A) structure was only 3.4%. A high proportion of the $\beta$-$O$-$4$/\alpha$-$O$-$4$ (A′) structure was also observed using LacR at pH 6.5. In the case of LacR at pH 6.5, the proportions of $\beta$-$5$ (B), $\beta\beta$ (C), $\beta$-$O$-$4$/\alpha$-$O$-$4$ (A′), and $\beta$-$O$-$4$ (A) structures were 46.6, 27.1, 26.4, and 1.9%, respectively. The proportions by LacR at pH 6.5 were similar to those obtained using LacT at pH 6.5. These results showed that the reaction pH was more critical for affecting the chemical structure of dehydrogenative polymerization products than the origin of laccase.

The dehydrogenative polymerization of CA using Rhus laccases under neutral conditions has been reported. Okusa et al. reported that Rhus laccase oxidized CA in acetone-water very slowly, and 20% of $\beta\beta$ dimer, 25% of $\beta$-$5$ dimer, and 2% of $\beta$-$O$-$4$ dimer were obtained at a reaction time of 144 h. Matsumoto et al. reported that DHP derived from CA contained the $\beta$-$5$ and $\beta\beta$ structures, but not the $\beta$-$O$-$4$ structure using Rhus laccase at pH 6.5 (Zutropf method). The reported high proportions of $\beta$-$5$ and $\beta\beta$ structures under neutral conditions were consistent with our results. However, the most significant differences from their results were that we observed a high proportion of the $\beta$-$O$-$4$/\alpha$-$O$-$4$ structure at pH 6.5 and a high proportion of the $\beta$-$O$-$4$ structure at pH 4.5 for both LacT and LacR. These results can be well explained by the reaction of $\beta$-$O$-$4$ type quinone methide as shown in Figure 5. It is reported that in the quinone methide reactions with vanillyl alcohol in aqueous solution, only the addition of water was observed at low pH, and the phenol addition was prominent under neutral conditions. A similar pH effect has been reported for the dehydrogenative polymerization of SA using HRP. These results suggest that in the biosynthesis of lignin, the type of laccase does not significantly affect the structure of lignin. It is likely that the surrounding environment during lignin biosynthesis, such as pH, has a greater influence on the structure of lignin. Because the high proportion of the $\beta$-$O$-$4$/\alpha$-$O$-$4$ structure is not observed for native lignins, lower pH is most likely a lignin biosynthesis condition as suggested by some studies on the reactivity of quinone methide and on the dehydrogenative polymerization of monolignol using HRP.

## CONCLUSIONS

The fungal laccase LacT and plant laccase LacR oxidized CA and SA at pH 4.5 and 6.5, respectively. However, DHP was obtained only from CA. Main lignin substructures, including $\beta$-$O$-$4$, $\beta$-$O$-$4$/\alpha$-$O$-$4$-$4$, $\beta\beta$, and $\beta$-$5$ structures, were observed in all DHPs. At pH 6.5, the $\beta$-$O$-$4$/\alpha$-$O$-$4$ (A′) structure was preferentially formed over the $\beta$-$O$-$4$ (A) structure for both LacT and LacR, which is different from the actual biosynthesis of lignin. In contrast, dehydrogenative polymerization products obtained at pH 4.5 by LacT and LacR had a much higher proportion of the $\beta$-$O$-$4$ (A) structure than the $\beta$-$O$-$4$/\alpha$-$O$-$4$ (A′) structure. The pH of the reaction solution had a greater effect on the structure of the resulting dehydrogenative polymerization products than the origin of laccase. These findings will contribute to our further understanding of the structure and biosynthesis of lignin.

## AUTHOR INFORMATION

### Corresponding Author

Takao Kishimoto — Bioorganic Chemistry Laboratory, Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, Imizu 939-0398, Japan; orcid.org/0000-0001-8340-2846; Phone: +81-766-56-7500 (ext. 1567); Email: takao@pu-toyama.ac.jp

### Authors

Ayumi Hiyama — Bioorganic Chemistry Laboratory, Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, Imizu 939-0398, Japan

Hiroshi Toda — Bioorganic Chemistry Laboratory, Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, Imizu 939-0398, Japan

Daisuke Urabe — Bioorganic Chemistry Laboratory, Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, Imizu 939-0398, Japan; orcid.org/0000-0002-1999-9374

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00144
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■ ABBREVIATIONS
CA, coniferyl alcohol; DHP, dehydrogenation polymer; HSQC, heteronuclear single quantum coherence; LacT, laccase from Trametes versicolor; LacR, laccase from Rhus vernicifera; NMR, nuclear magnetic resonance; SA, sinapyl alcohol

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