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# Purification and Characterization of Laccase from Basidiomycete *Fomitella fraxinea*

**Park, Kyung Mi[†] and Sang-Shin Park***

*Department of Biotechnology, Dongguk University, Gyeongju 780-714, Korea*

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**A laccase was isolated from the culture filtrate of the basidiomycete *Fomitella fraxinea*. The enzyme was purified to electrophoretical homogeneity using ammonium sulfate precipitation, anion-exchange chromatography, and gel-filtration chromatography. The enzyme was identified as a monomeric protein with a molecular mass of 47 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel-filtration chromatography, and had an isoelectric point of 3.8. The N-terminal amino acid sequence for the enzyme was ATXSNXKTLAAD, which had a very low similarity to the sequences previously reported for laccases from other basidiomycetes. The optimum pH and temperature for 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) were 3.0 and 70°C, respectively. The enzyme also showed a much higher level of specific activity for ABTS and 2,6-dimethoxyphenol (DMP), where the $K_m$ values of the enzyme for ABTS and 2,6-DMP were 270 and 426 μM, respectively, and the $V_{max}$ values were 876 and 433.3 μM/min, respectively. The laccase activity was completely inhibited by L-cysteine, dithiothreitol (DTT), and sodium azide, significantly inhibited by $Ni^+$, $Mn^{2+}$, and $Ba^{2+}$, and slightly stimulated by $K^+$ and $Ca^{2+}$.**

**Keywords:** *Fomitella fraxinea*, laccase, purification, characterization

Lignin, the most abundant aromatic polymer in nature, is produced by plants from polysaccharides, including cellulose and hemicellulose. The degradation of lignin is carried out by a group of basidiomycetes categorized as white-rot fungi. It is well known that white-rot fungi produce extracellular lignin-degrading enzymes, including lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase [33]. Although the production of MnP and laccase is much more common than that of LiP and MnP, the white-rot basidiomycete *Coriolopsis rigida* secretes no detectable LiP or MnP, yet a good amount of laccase [28], and this laccase has been demonstrated to be capable of degrading lignin, including nonphenolic moieties. Additional studies have also showed that laccase plays a key role in the lignin degradation process [22].

Laccase (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2) is a polyphenol oxidase that catalyzes the oxidation of phenolic compounds and aromatic amines with molecular oxygen as the electron acceptor [31]. Laccases are typically multicopper blue oxidases containing 2–4 copper atoms per molecule and are widely distributed in many plants and fungi. In the presence of an appropriate redox mediator, such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) or 1-hydroxybenzotrizole (HBT), laccase also catalyzes the oxidation of nonphenolic lignin model compounds [2] and degrades polycyclic aromatic hydrocarbons [27] and various dye pollutants. Therefore, these enzymes have recently been attracting broad attention owing to their potential industrial application in biopulping, textile dye bleaching, the degradation of aromatic pollutants, and detoxification of polluted water [7, 13, 19, 30]. Thus, fungal strains with a high level of enzymatic activity and substrate affinity are essential for the further application of biotechnology.

Laccases have already been purified and characterized from the mycelia and fruit bodies of several basidiomycetes, including *Trametes versicolor, Lentinus edodes*, *Agaricus bisporus*, and *Pleurotus ostretus* [3, 21, 23, 26]. Additionally, certain fungal laccase genes have also been cloned [11, 17]. However, the mechanism of lignin degradation by laccase remains to be defined. Therefore, the purification and characterization of laccases from novel wood-rotting fungi will help elucidate the mechanism and effectiveness of the enzyme for industrial application.

The basidiomycete *Fomitella fraxinea*, which is also a white-rot fungus, has primarily been investigated for its

*Corresponding author
Phone: 82-54-770-2225; Fax: 82-54-770-2210;
E-mail: sspark@dongguk.ac.kr

[†]Present address: Division of Biotechnology R&BD, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-333, Korea

pharmaceutical activities as an immunostimulating agent, antioxidant, and fibrinolytic protease [8, 15, 25]. However, there has been almost no other basic research on this mushroom. In a previous report, the current authors investigated the optimal production of laccase in a culture of *Fomitella fraxinea* [24]. Accordingly, this study describes the purification and characterization of the laccase from the culture supernatant of *F. fraxinea*.

## MATERIALS AND METHODS

### Microorganism and Culture Conditions

The *F. fraxinea* strain (ASI-17015) was obtained from the National Institute of Agricultural Science and Technology (NIAST) in Korea. The fungus was grown on a potato dextrose agar plate at 25°C for 7 days, and maintained at 4°C. For laccase production, the fungus was inoculated into a medium supplemented with 2% dextrose, 0.4% $(NH_4)_2HPO_4$, 0.05% $Na_2HPO_4{\cdot}7H_2O$, and 0.05% KCl, and cultured at 25°C for 10 days on a rotary shaker, as described previously [24]. The resulting culture supernatant was then used as the source of the enzyme to carry out the purification and characterization in this study.

### Enzyme and Protein Assays

The laccase activity was determined using ABTS as the substrate. The assay mixture contained 5 mM ABTS, a 100 mM sodium acetate buffer (pH 5.0), and 100-µl aliquots of an appropriately diluted enzyme solution. The oxidation of ABTS was monitored spectrophotometrically by measuring the increase in the $A_{420}$ ($\varepsilon$= 36,000 $M^{-1}cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmole of ABTS per minute at 25°C. The protein concentration was estimated using a Bradford assay [4], with bovine serum albumin as the standard.

### Laccase Purification

Unless otherwise stated, all the procedures were performed at 4°C. The culture fluid was filtered through a Whatman No. 1 filter paper and centrifuged at 10,000 ×*g* for 10 min. Ammonium sulfate was added to the supernatant to give an 80% saturation, and the precipitated proteins were collected by centrifugation at 10,000 ×*g* for 30 min. The precipitate was then dissolved in an appropriate volume of a 50 mM sodium acetate buffer (pH 6), dialyzed overnight against the same buffer, and concentrated by ultrafiltration using a YM10 membrane (Amicon Corp., U.S.A.). Next, the concentrated proteins were applied to an ion-exchange DEAE-Sepharose FF column (2.5×30 cm; Amersham Biosciences, Sweden) previously equilibrated with a 50 mM sodium acetate buffer (pH 6.0), and the bound proteins eluted using a linear gradient of 0 to 0.5 M NaCl in the same buffer at a flow rate of 2.5 ml/min. The fractions containing laccase activity were pooled and concentrated in an Amicon stirred cell using a YM-10 membrane. The concentrate was then loaded onto a Superdex 200 HR gel filtration column (1.0× 60 cm; Amersham Biosciences) preequilibrated with a 50 mM sodium phosphate buffer (pH 6.0) and eluted with the same buffer at a flow rate of 0.5 ml/min using an FPLC (fast protein liquid chromatography; Amersham Biosciences) system. The fractions containing laccase activity were pooled, concentrated, and further purified by FPLC with a Mono Q column (0.5×5 cm; Amersham Biosciences) using the same buffer and linear gradient of 0–0.45 N NaCl at a flow rate of 0.5 ml/min. The fractions containing laccase activity were collected, concentrated, and used as the purified enzyme preparation.

### Molecular Mass Determination

The molecular mass of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration chromatography on a Sephacryl S-200 column. The SDS-PAGE was performed with 12% polyacrylamide gels using the method described by Laemmli [14]. The molecular mass markers used were phosphorylase b (108 kDa), bovine serum albumin (98 kDa), ovalbumin (54 kDa), carbonic anhydrase (33 kDa), and a soybean trypsin inhibitor (29 kDa). After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB) R-250.

### Isoelectric Focusing

The isoelectric point of the enzyme was determined using a Mini IEF cell (Model 111; Bio-Rad Laboratories) with a 5% polyacrylamide gel, ampholyte (pH 3–5), and low range markers (Amersham Biosciences) as the standards.

### N-Terminal Amino Acid Sequence Analysis

After the SDS-PAGE, the purified enzyme on the gel was transferred to a polyvinylidene difluoride (Sesqui-Blot PVDF; Bio-Rad) membrane by electroblotting and stained with CBB. The stained band was excised and analyzed by the automated Edman degradation method using a Procise 491 HT protein sequencer (Applied Biosystems, U.S.A.).

### Substrate Specificity and Determination of Kinetic Constants

The oxidation of substrates by the purified laccase was determined spectrophotometrically at the specific wavelength of each substrate. The assays were performed by measuring the increase in the $A_{420}$ for ABTS, $A_{470}$ for 2,6-dimethoxyphenol (DMP) ($\varepsilon$=35,645 $M^{-1}cm^{-1}$), $A_{530}$ for syringaldazine ($\varepsilon$=65,000 $M^{-1}cm^{-1}$), and $A_{470}$ for guaiacol ($\varepsilon$=12,000 $M^{-1}cm^{-1}$) in a 100 mM sodium acetate buffer (pH 5.0). The kinetic constants, $K_m$ and $V_{max}$, of the enzyme were determined using a Lineweaver-Burk plot with ABTS and 2,6-DMP as the substrates in a 100 mM sodium acetate buffer (pH 5.0).

### pH and Temperature Dependence

The optimum pH for the laccase was estimated using ABTS as the substrate in a 100 mM sodium citrate buffer (pH 2.5–6.0) and 100 mM sodium phosphate buffer (pH 6.5–8.0). The effect of pH on the enzyme stability was measured after 1 h of incubation at various pHs at 25°C. The optimum temperature for the laccase was determined by measuring the enzyme activity at various temperatures ranging from 20°C to 90°C in a 100 mM sodium acetate buffer (pH 5.0). The effect of temperature on the enzyme stability was investigated by incubating the enzyme solution for 1 h in a 100 mM sodium acetate buffer (pH 3.0) at various temperatures. After incubation, the remaining activity was determined.

### Effect of Metal Ions and Potential Inhibitors

The activity of the laccase was measured using ABTS in the presence of several metal ions, including 1 mM of $Ni^{2+}$, $K^+$, $Ca^{2+}$,

**Table 1.** Purification table for laccase from *F. fraxinea*.

| Purification step | Volume (ml) | Total protein (mg) | Total activity (unit) | Specific activity (unit/mg) | Recovery (%) | Purification fold |
|---|---|---|---|---|---|---|
| Crude extract | 1,200.0 | 315.4 | 507.7 | 1.6 | 100 | 1 |
| Ammonium sulfate precipitation | 34.0 | 54.6 | 152.8 | 2.8 | 30.1 | 1.8 |
| DEAE-Sepharose FF chromatography | 11.5 | 18.2 | 145.8 | 8.0 | 28.7 | 5.0 |
| Superdex-200 chromatography | 3.4 | 5.8 | 115.4 | 19.9 | 22.7 | 12.3 |
| Mono-Q chromatography | 1.8 | 0.6 | 56.0 | 93.3 | 11.0 | 58.0 |

$Cu^{2+}$, $Al^{2+}$, $Mg^{2+}$, $Mn^{2+}$, and $Ba^{2+}$. The effect of potential inhibitors on the enzyme activity was determined in the presence of the following substances: ethylenediaminetetraactic acid (EDTA), dithiothretiol (DTT), $NaN_3$, dimethylsulfoxide (DMSO), and SDS. These assays were also performed with ABTS as the substrate in a 100 mM sodium acetate buffer (pH 5.0).

## Results and Discussion

### Laccase Purification

The laccase was purified to homogeneity from the culture filtrate of *F. fraxinea* mycelia, and the purification steps are summarized in Table 1. After the three chromatography steps, the enzyme was purified 58-fold, with an overall yield of 11.0%. The elution profile from the ion-exchange chromatography on the Mono-Q column showed the laccase activity as a single peak (Fig. 1), indicating that the *F. fraxinea* laccase did not include any isozymes, in contrast to the laccases of *Pleurotus eryngii*, *Ganoderma lucidum*, and *Trametes versicolor*, which have two or three forms of isozyme [6, 12, 20]. The purified laccase also appeared as a single protein band on the SDS-PAGE gel (Fig. 2A) and exhibited the same band location as previously reported for gel activity staining [24].

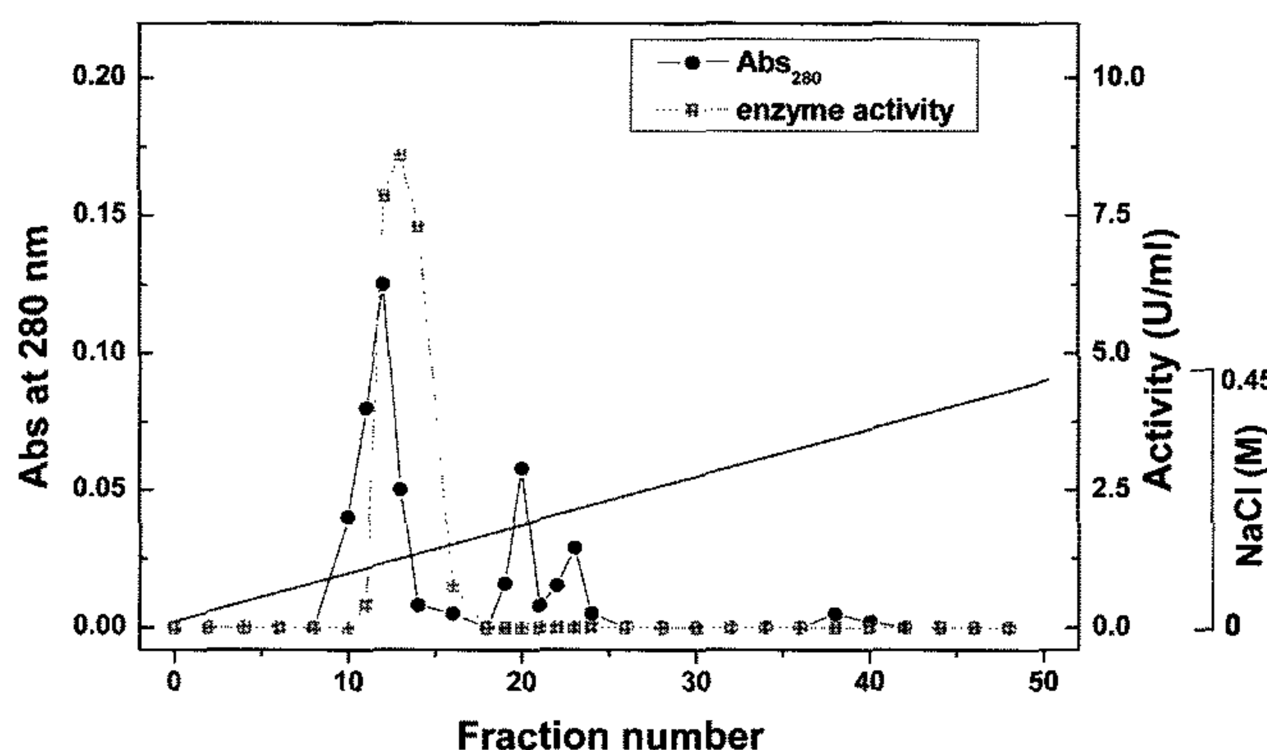


**Fig. 1.** Elution profile for laccase from *F. fraxinea* based on anion-exchange chromatography with a Mono-Q column (0.5× 5 cm).

The enzyme was eluted with a 50 mM sodium phosphate buffer (pH 5.0) at a flow rate of 0.5 ml/min, using FPLC.

### Molecular Mass and Isoelectric Point

The molecular mass of the enzyme was 47 kDa, as determined by SDS-PAGE (Fig. 2A), and 47.6 kDa, as estimated by gel filtration chromatography on a Sephacryl S-200 column (data not shown). The results suggested that the enzyme is a monomeric protein similar to those of most other fungal laccases. Most fungal laccases are monomeric proteins with molecular masses between 50 and 80 kDa [1]. Thus, the laccase from *F. fraxinea* was clearly slightly smaller than those of other species of basidiomycetes. The isoelectric point for the laccase was 3.8, as determined by analytical isoelectric focusing (Fig. 2B). Many other laccases from basidiomycetes, such as *Agaricus blazei*, *P. eryngii*, *L. edodes*, *C. rigida*, and *Pycnoporus cinnabarinus*, have acidic isoelectric points ranging from 3.0 to 4.8 [9, 20, 21, 28]. Therefore, this study also identified that the pI of *F. fraxinea* laccase was within an acidic range, like those of other fungal laccases and especially similar to the pI (3.7) of the laccase from *P. cinnabarinus* and pI (3.9) of the enzyme from *C. rigida*.

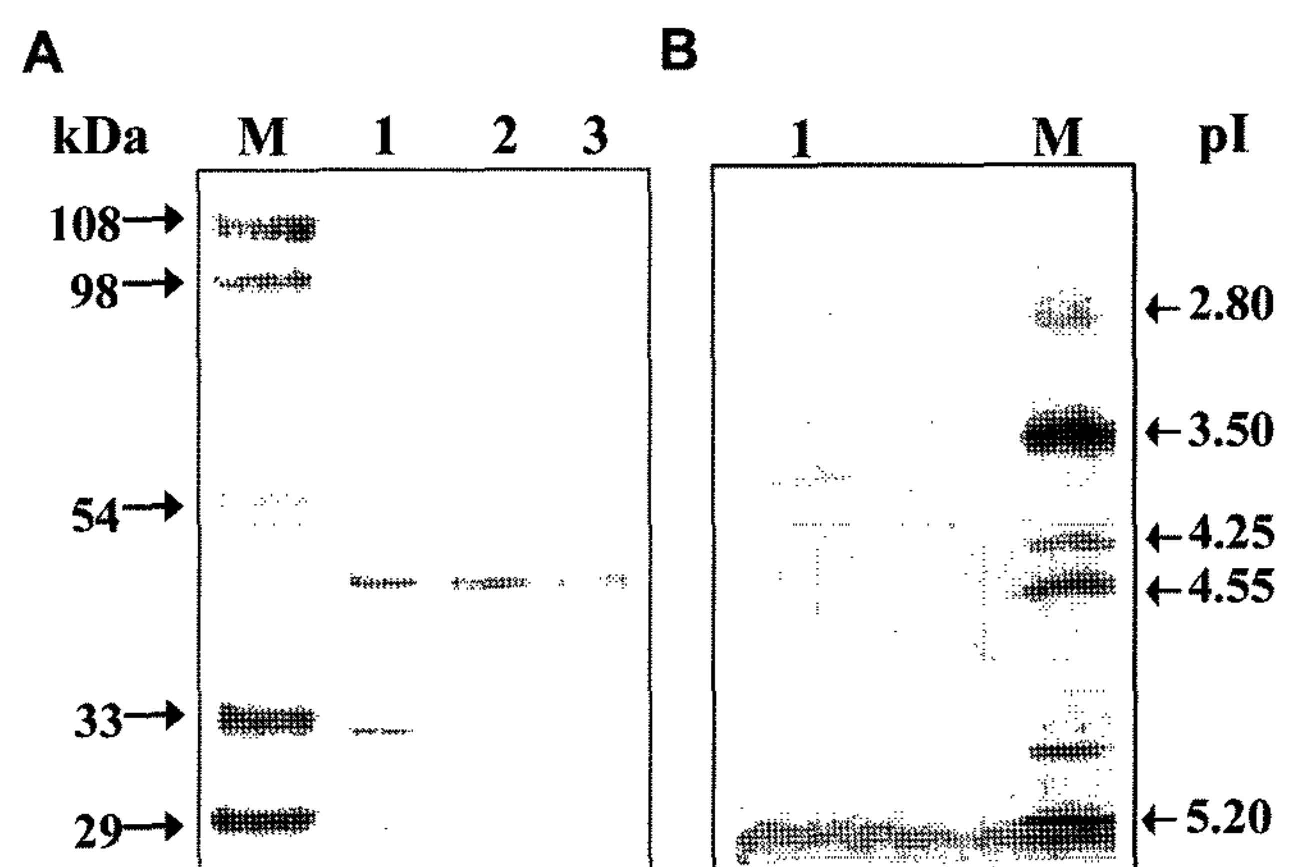


**Fig. 2.** Electrophoresis of *F. fraxinea* laccase.

**A.** SDS-PAGE of purified enzymes. Lanes: M, molecular mass markers: phosphorylase B (108 kDa), bovine serum albumin (98 kDa), ovalbumin (54 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (29 kDa); 1, fraction from DEAE-Sepharose FF ion-exchange chromatography; 2, fraction from Superdex-200 gel-filtration chromatography; 3, purified enzyme from Mono-Q ion-exchange chromatography. **B.** Isoelectric focusing of enzyme. Lanes 1, purified enzyme; M, pI marker proteins.

**Table 2.** N-terminal sequences for *F. fraxinea* laccase and other fungal laccases.

| Microorganism | N-Terminal sequence |
|---|---|
| *Fomitella fraxinea* | A T - S N - K T L A A D |
| *Pleurotus eryngii* [20] | A T K K L - D F H I I N |
| *Lentinus edodes* [21] | A I G P V T D L H I V N |
| *Agaricus bisporus* [26] | K T R - T F D F D L V N |
| *Coriolus hirsutus* [29] | A I G P T A D L T I S N |
| *Pycnoporus cinnabarinus* [9] | A I G P V A D L T L T N |
| *Ceriporopsis subvermispora* [10] | A I G P V T D L E I T D |

Identical corresponding amino acid residues are underlined.

### Analysis of N-Terminal Amino Acid Sequence

The N-terminal amino acid sequence for the laccase of *F. fraxinea* is shown in Table 2, along with the sequences determined for laccases from other basidiomycetes. The sequence of the enzyme was determined up to 12 amino acids as ATXSNXKTLAAD. However, since the enzyme showed a significantly low homology with other fungal laccases [9, 10, 20, 21, 26, 29], it is suggested that the laccase of *F. fraxinea* is quite different from those of other basidiomycetes and may be encoded by a different gene.

### Substrate Specificity and Kinetic Constants

The substrate specificity of the purified laccase was examined with various phenolic and nonphenolic compounds, such as monoaromatic phenolic substrates (2,6-DMP, guaiacol), a complex phenol (syringaldazine), and nonphenolic heterocyclic compound (ABTS). The enzyme showed the highest level of activity with ABTS at 93.3 U/ml. The enzyme also displayed a much higher level of activity with 2,6-DMP (87 U/ml), which includes a double methoxy group, than with guaiacol, which only includes one methoxy group (45 U/ml). Meanwhile, the enzyme exhibited a very low activity with syringaldazine. The apparent $K_m$ and $V_{max}$ values of the enzyme for ABTS and 2,6-DMP were determined from a Lineweaver-Burk plot (Table 3), where the $K_m$ values were 270 and 426 μM, respectively, and the $V_{max}$ values were 876 and 433.3 μM/min, respectively. In addition, the $k_{cat}$ values for both substrates were 208 and 103.2 $s^{-1}$, respectively, whereas the catalytic efficiencies ($k_{cat}/K_m$) of the enzyme for the substrates were $7.7\times10^5$ and $2.4\times10^5$ $s^{-1}\cdot M^{-1}$, respectively, indicating that the enzyme had a higher affinity and catalytic efficiency toward ABTS than 2,6-DMP. Fungal laccases are known to posses a wide range of substrate affinities [18, 21], where the highest affinity is usually for ABTS, although *Phellinus ribis*, *Rigidoporus lignosus*, and *A. blazei* all have a higher affinity for syringaldazine [5, 18, 32]. However, in this study, the substrate specificity of the laccase from *F. fraxinea* was similar to that of many other fungal laccases, and the $k_{cat}$ value of the enzyme for ABTS was even higher than those of the laccases from *Ceriporiopsis subvermispora*, *Trametes villosa*, *P. cinnabarinus*, and *A. blazei* [16], suggesting that ABTS may be an effective mediator for this enzyme.

**Table 3.** Kinetic constants for laccase from *F. fraxinea*.

| Substrate | $K_m$ (μM) | $V_{max}$ (μM·min$^{-1}$) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}\cdot M^{-1}$) |
|---|---|---|---|---|
| ABTS[a] | 270.0 | 876.0 | 208.0 | $7.7\times10^5$ |
| 2,6-DMP[b] | 426.0 | 433.3 | 103.2 | $2.4\times10^5$ |

[a]2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonate).
[b]2,6-Dimethoxyphenol.

### Effect of Metal Ions and Inhibitors

The effect of metal ions on the laccase activity was tested using ABTS as the substrate (Table 4). The enzyme was strongly inhibited by 1 mM of $Ni^{2+}$ (57% inhibition), $Al^{2+}$ (43% inhibition), $Mn^{2+}$ (51% inhibition), and $Ba^{2+}$ (52% inhibition), slightly stimulated by 1 mM of $K^+$ and $Ca^{2+}$, and essentially unaffected by 1 mM of $Cu^{2+}$. Similar

**Table 4.** Effect of metal ions on the activity of *F. fraxinea* laccase.

| Metal ion | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | - | 100 |
| $NiCl_2$ | 1 | 42.9 |
| KCl | 1 | 119.1 |
| $CaCl_2$ | 1 | 114.4 |
| $CuSO_4$ | 1 | 85.8 |
| $CuCl_2$ | 1 | 91.3 |
| $AlCl_2$ | 1 | 57.1 |
| $MgCl_2$ | 1 | 81.0 |
| $MnCl_2$ | 1 | 49.0 |
| $BaCl_2$ | 1 | 48.2 |

**Table 5.** Effect of various inhibitors and organic solvents on the activity of *F. fraxinea* laccase.

| Inhibitor | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | - | 100 |
| DTT[a] | 0.1 | 2.2 |
| | 0.5 | 0 |
| $NaN_3$ | 0.1 | 2.8 |
| | 0.5 | 0 |
| L-Cysteine | 0.1 | 5.3 |
| | 0.5 | 0 |
| EDTA[b] | 1 | 98.9 |
| | 10 | 64.3 |
| DMSO[c] | 1 | 92.3 |
| | 10 | 65.7 |
| SDS[d] | 1 | 99.4 |
| | 10 | 78.6 |

[a]Dithiothreitol.
[b]Ethylenediaminetetraacetic acid.
[c]Dimethylsulfoxide.
[d]Sodium dodecyl sulfate.

results were also previously reported for the laccase from *Perenniporia tephropora*, which was strongly inhibited by $Mn^{2+}$ and $Ni^{2+}$, yet unaffected by $Cu^{2+}$ [34]. The sensitivity of the enzyme towards several putative laccase inhibitors was also tested (Table 5), where the laccase was completely inhibited by 0.5 mM sodium azide, the most effective inhibitor of many oxidative enzyme reactions, 0.5 mM L-cysteine, a classical inhibitor of phenol oxidase-type activity, and 0.5 mM DTT. Conversely, EDTA, DMSO, and SDS had a relatively low inhibitory effect, even at a higher concentration (10 mM). These results were also very similar to those previously reported for the laccases from *C. hirsutus* [29], *P. cinnabarinus* [9], and *L. edodes* [21].

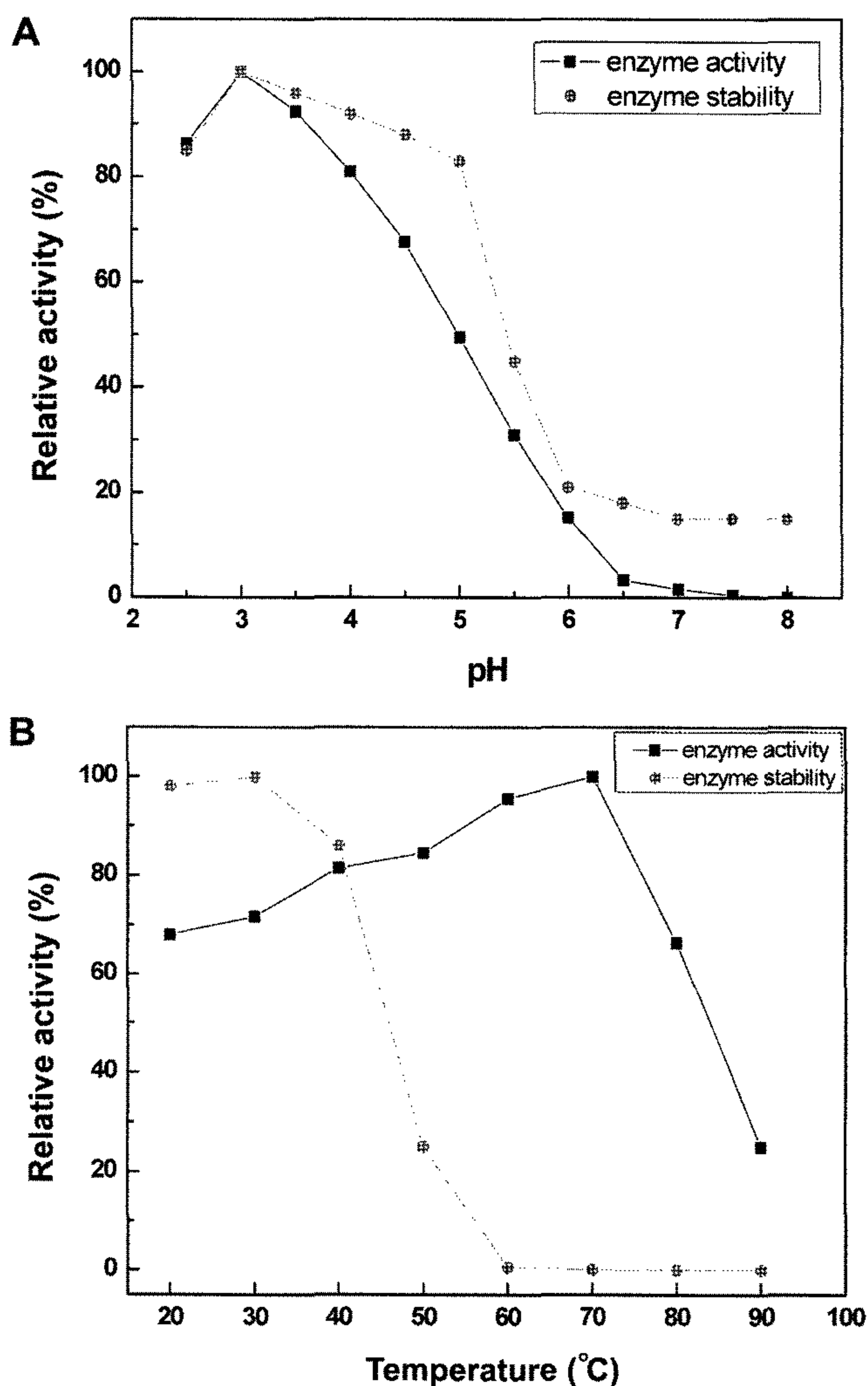


**Fig. 3.** Effect of pH (**A**) and temperature (**B**) on the activity of *F. fraxinea* laccase.

The enzyme activity was assayed within a pH range of 2.5–8.0 (0.1 M sodium citrate buffer for pH 2.5–6.0 and 0.1 M sodium phosphate buffer for pH 6.5–8.0) at 25°C. The effect of pH on the enzyme activity was measured by incubating the enzyme for 1 h at various pHs at 25°C. The enzyme activity was also assayed at various temperatures from 20 to 90°C in a 0.1 M sodium acetate buffer (pH 3.0), and the enzyme stability measured after 1 h of incubation at the various temperatures at pH 3.0.

**Effect of pH and Temperature on Enzyme Activity and Stability**

The effect of pH on the enzyme activity was investigated at pH values ranging from 2.5 to 8.0 with ABTS as the substrate (Fig. 3A). The optimum pH for the enzyme was identified as 3.0, which was consistent with the optimum pH for the laccase isozyme from *C. subvermispora* and enzyme from *C. hirsutus* [10, 29]. Other studies have also reported very low optimal pHs (between 3.0 and 5.7) for fungal laccases, except for the laccase from *Rhizoctonia praticola*, which exhibited a neutral optimal pH with various substrates [1]. When the effect of pH on the enzyme stability was examined at 25°C for 1 h, the enzyme remained stable within an acidic pH range from 3.0 to 5.0. Meanwhile, the optimum temperature of the laccase for ABTS oxidation was 70°C (Fig. 3B), which was much higher than the optimum temperatures previously reported for other fungal laccases, ranging from 40°C to 50°C [21, 29, 33]. The thermal stability of the enzyme was also determined by incubating the enzyme at pH 3.0 for 1 h. The enzyme remained stable up to 40°C, yet the stability decreased rapidly above 40°C, which was similar to the results previously reported for the laccases from *L. edodes* and *A. blazei* [21, 32]. However, the enzyme was less stable than the laccases from *P. ribis* and *Trametes* sp. strain AH28-2, which remained stable at 55°C and 70°C for more than 1 h, respectively [18, 33].

Therefore, this study found that the *F. fraxinea* laccase was slightly smaller than most other fungal laccases and had quite a different N-terminal amino acid sequence. Furthermore, the enzyme was very active at a relatively acidic pH and higher temperature when compared with other laccases. In conclusion, the present results showed that the laccase from the basidiomycete *F. fraxinea* is a new member of a growing family of laccase enzymes that possess important properties for industrial application. In view of the importance of laccase, the structure and catalytic mechanism of the *F. fraxinea* laccase will be studied further.

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