5. Purification and characterization of laccases

5.1 Effect of glucose concentration on laccase production

Laccase could be regulated to be the only lignin-degrading enzyme that was produced by *Ganoderma* sp. KU-Alk4 in Kirk's medium by shifting the medium pH to 8.0. In these conditions no activities of LiP or MnP were detected. Laccase production started on day 3 on the addition of 0.85 mM veratryl alcohol as inducer (Figure 46). Higher activity of laccase was detected in G1% than in G4% culture. The fungus produced overall laccase activity (U/mL) about 2 fold higher in G1% medium than in G4%. However, the mycelial dry weight of fungus in G1% medium was 1.6 fold lower than that in G4% at day 9, the time at which the maximal laccase activities were observed. The specific activities of laccase at day 9 in G1% and G4% media were 126.8 U/mg cell dry weight and 33.3 U/mg cell dry weight, respectively.

Zymograms of laccase isozymes in the crude enzymes collected at day 9 from G1% and G4% cultures (Figure 47) showed two major active bands reacting with DMP (KULac 1 and 2) in G1% culture (Figure 47A). Three major active bands were observed from G4% cultures (KULac 3, 4 and 5; Figure 47B). The 5 isozymes showed different molecular weights. From the results, lignin-degrading enzyme production by this new isolate, *Ganoderma* sp. KU-Alk4, was controlled by the initial glucose concentration which affected not only to the total amount of laccase activity but also regulated the production of different isozymes.

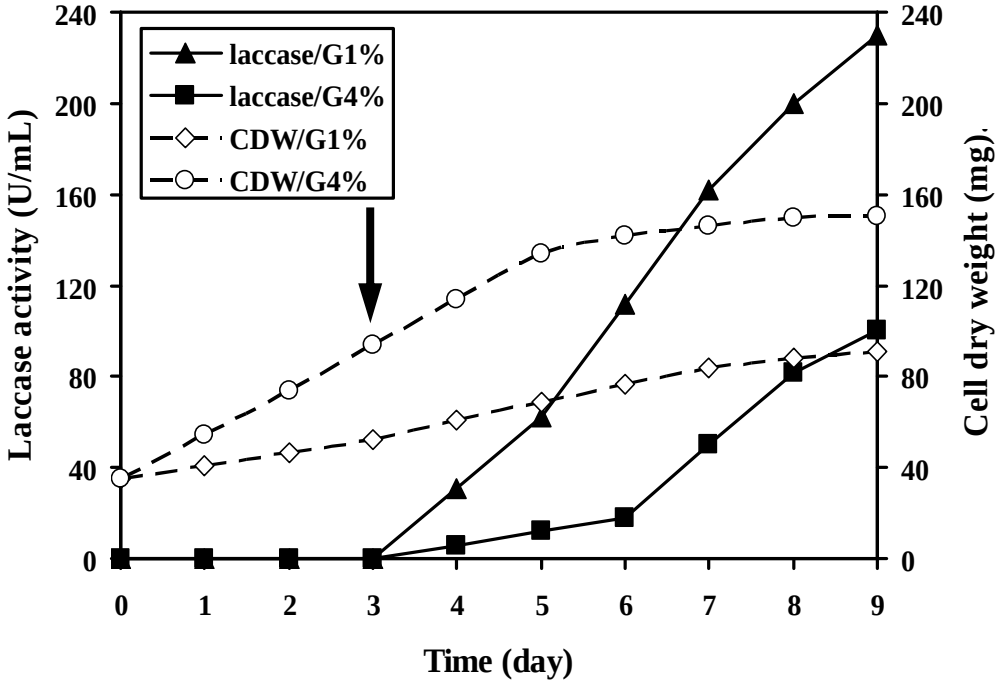

Figure 46 Effect of glucose concentrations on laccase production and cell dry weight (CDW) of *Ganoderma* sp. KU-Alk4. The cultures grown in G1% and G4% Kirk media supplemented with 0.85 mM veratryl alcohol as inducer. Arrow indicates the time of the inducer addition. Values are the average of three independent experiments and the maximal mean deviation is ± 8% of values.

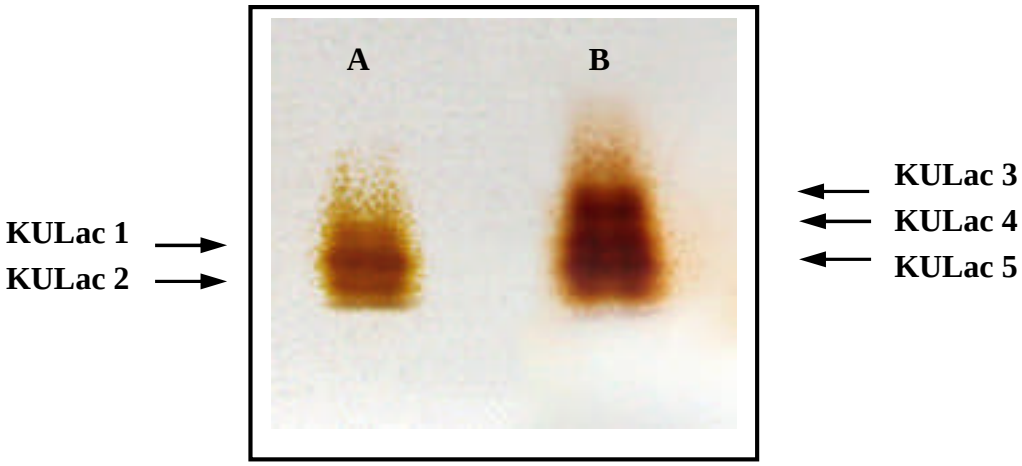

Figure 47 Zymograms of laccase isozymes monitored in the crude enzyme of *Ganoderma* sp. KU-Alk4 grown in G1%, (A), and G4%, (B), Kirk's media. Samples containing 1.5 U of laccase activity collected at 9 days were used.

5.2 Purification of laccase isozymes

Table 25 summarizes the purification steps of KULac 1 and 2 from G1% culture. KULac 1 from G1% culture was purified 4 fold giving specific activity 6,133 U/mg protein. Purified KULac 2 obtained from G1% culture had specific activity of 4,205 U/mg protein. After purification, KULac 1 showed 70% of the initial activity and KULac2 was 2%. Protein and activity profiles from DEAE-Toyopearl column II chromatograph showed that KULac 1 was the main isozyme and eluted at 0.23 M NaCl, while KULac 2 was the minor one that eluted at 0.37 M NaCl (Figure 48).

Table 26 summarized the purification steps of KULac 3, 4 and 5 from G4% culture. Purified KULac 3 that was eluted from the second DEAE-Toyopearl column chromatograph at 0.14 M NaCl (Figure 49) comprised 53% of the initial activity and had a specific activity of 3,796 U/mg protein. Very small amounts of KULac 4 eluted at 0.23 M NaCl, were obtained (less than 1%) and its specific activity was 2,652 U/mg protein. KULac 5 was eluted by 0.18 M NaCl, showed 24% of the initial activity and had the highest specific activity 4,918 U/mg protein. Purification fold of KULac 3, 4 and 5 was 6.7, 4.7 and 8.7 fold, respectively (Table 26). The protein profile suggests that *Ganoderma* sp. KU-Alk4 secreted laccase as dominant proteins in the cultivated conditions.

Table 25 Purification steps of laccase isozymes from *Ganoderma* sp. KU-Alk4 cultured in G1% Kirk's medium

Purification step	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg Protein)	Yield (%)	Purification (fold)
Crude enzyme	120.0	$200.0x10^3$	1666.7	100.0	1.0
Ultrafiltration	77.5	$196.0x10^3$	2528.2	98.0	1.5
DEAE-Toyopearl I	41.5	$164.0x10^3$	3955.6	82.0	2.4
Dialysis&Ultrafiltration	41.5	$164.0x10^3$	3960.2	82.0	2.4
DEAE-Toyopearl II					
KULac 1	22.8	$140.0x10^3$	6132.9	70.0	3.7
KULac 2	1.1	$4.7x10^3$	4204.6	2.3	2.5

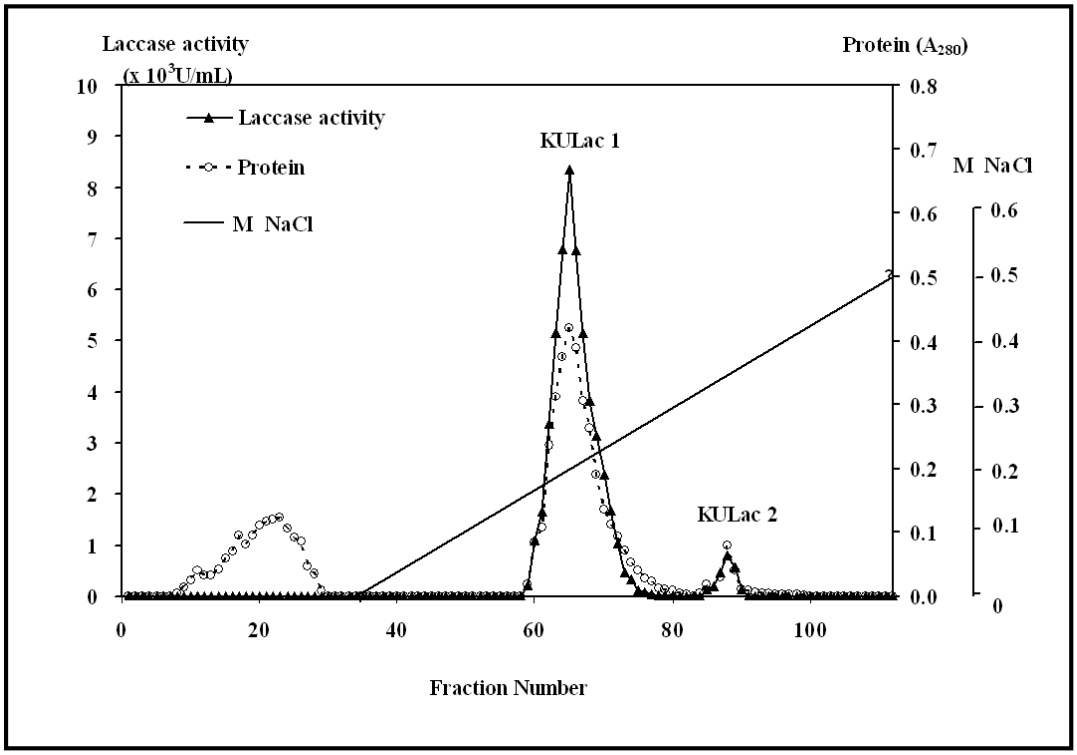

Figure 48 Protein and activity profiles of laccase isozymes of *Ganoderma* sp. KU-Alk4 grown in 1% glucose medium, from DEAE-Toyopearl column (10 X 50 mm), 50 mM Tris-HCl buffer, pH 7.5 with 0-0.6 M NaCl as an elution buffer, flow rate 5 mL/h and 1.5 mL fractions were collected.

Table 26 Purification steps of laccase isozymes from *Ganoderma* sp. KU-Alk4 cultured in G4% Kirk's medium

Purification step	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg Protein)	Yield (%)	Purification (fold)
Crude enzyme	168.0	95.2×10^3	566.7	100.0	1.0
Ultrafiltration	76.3	93.6×10^3	1226.6	98.3	2.2
DEAE-Toyopearl I	35.4	82.4×10^3	2324.2	86.5	4.1
Dialysis&Ultrafiltration	35.2	82.4×10^3	2338.0	86.5	4.1
DEAE-Toyopearl II					
KULac 3	13.4	50.8×10^3	3796.3	53.4	6.7
KULac 4	0.3	0.8×10^3	2652.0	0.8	4.7
KULac 5	4.7	22.9×10^3	4918.4	24.0	8.7

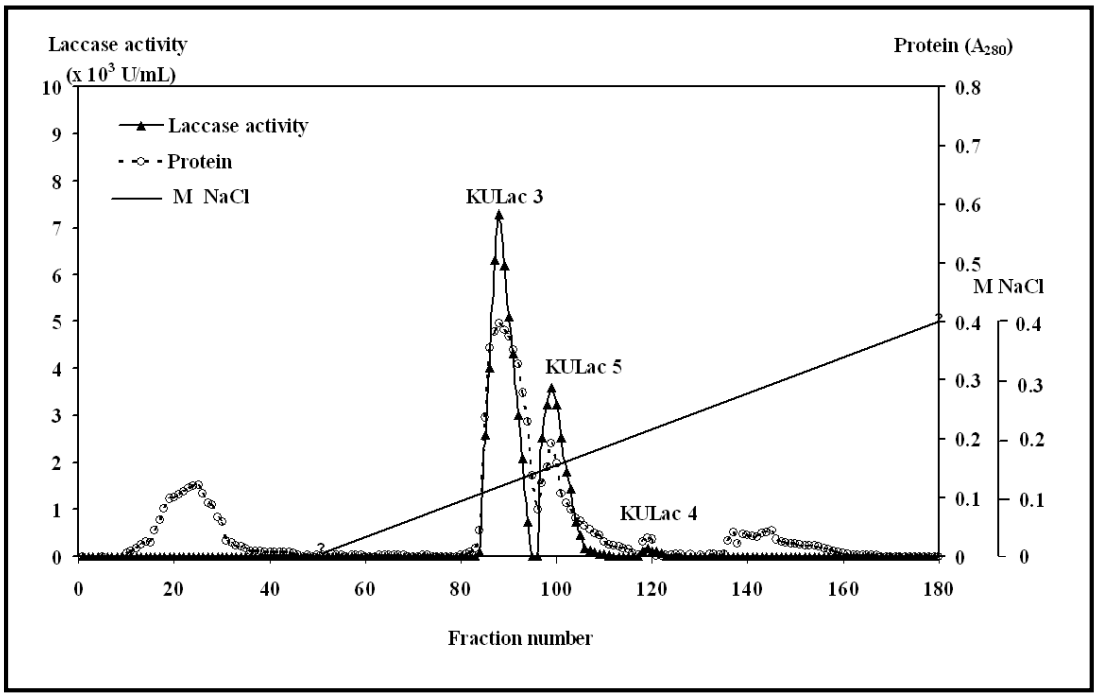

Figure 49 Elution profile of KULac 3, KULac 4 and KULac 5 from G4% culture of *Ganoderma* sp. KU-Alk4 on DEAE-Toyopearl column (10 X 50 mm), 50 mM Tris-HCl buffer, pH 7.5 with 0-0.4 M NaCl as an elution buffer, flow rate 5 mL/h and 1.5 mL fractions were collected.

5.3 Characterization of the purified laccase isozymes (KULacs)

5.3.1 Molecular weight and numbers of subunit

The molecular weights of KULac 1, 2, 3, 4 and 5 on SDS-PAGE were 67, 50, 71, 47 and 65 kDa, respectively (Figure 50) The native protein of each isozyme on 10% non-denaturing native-PAGE showed that KULac 1, 2, 3, 4 and 5 had molecular masses of 90, 53, 112, 100 and 74 kDa, respectively (Figure 51). Therefore, KULac 4 might be composed of 2 subunits of the same molecular weight, whereas KULac 1, 2, 3 and 5 were homogeneous proteins. This confirms that laccases from basidiomycetes, including *G. lucidum*, are generally monomeric proteins (Yaropolov *et al*., 1994; Ko *et al*., 2001). Most fungal laccases have molecular weights ranging from 59-110 kDa, mostly 66 kDa (Luisa *et al*., 1996; Wesenberg *et al*., 2003). Ko *et al*. (2001) reported that *G. lucidum*, a Korean isolate, produces 3 laccase isozymes in a complete medium without any specific induction, whose molecular weights were between 65 kDa and 68 kDa. D'Souza *et al*. (1999) also reported 5 major laccase isozymes produced by *G. lucidum* in high nitrogen medium, 2 of which were 40 kDa and 66 kDa.

Our *Ganoderma* sp. KU-Alk4 produced different types and numbers of laccase isozymes. Two isozymes (KULac 1 and 2) and 3 different isozymes (KULac 3, 4, 5) were produced in G1% and G4% medium, respectively. Molecular weights of the isozymes that were produced in G1% medium were lower than that in G4% medium. It is typical of white-rot fungi to produce multiple isozymes of laccase. A maximum number of 10 isozymes was reported in *Flavodon flavus* (Raghukumar *et al*., 1999) but there was no evidence showing that concentration of glucose could regulate the production of different types of laccases. This work, to our knowledge, is the first report that showed the effect of glucose concentration that influenced on the fungal physiology on laccase production to produce different sizes of isozymes.

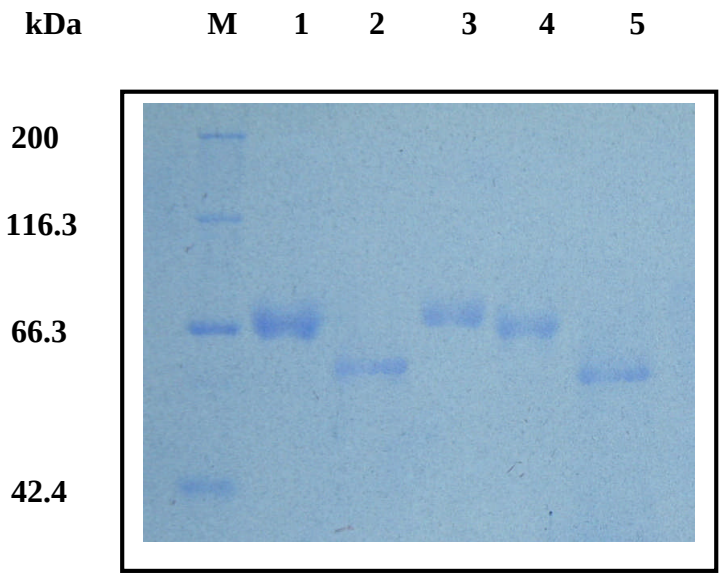

Figure 50 SDS-PAGE of the purified laccase isozymes from *Ganoderma* sp. KU-Alk4. Samples containing 2 µg of protein. Lane *1*: KULac 1, lane *2*: KULac 2, lane *3*: KULac 3, lane *4*: KULac 5, lane *5*: KULac 4. Lanes *M*: Molecular weight markers were myosin, 200.0 kDa; *β*-galactosidase, 116.3 kDa; albumin, 66.3 kDa and aldolase, 42.4 kDa (DAIICHI Pure Chemicals).

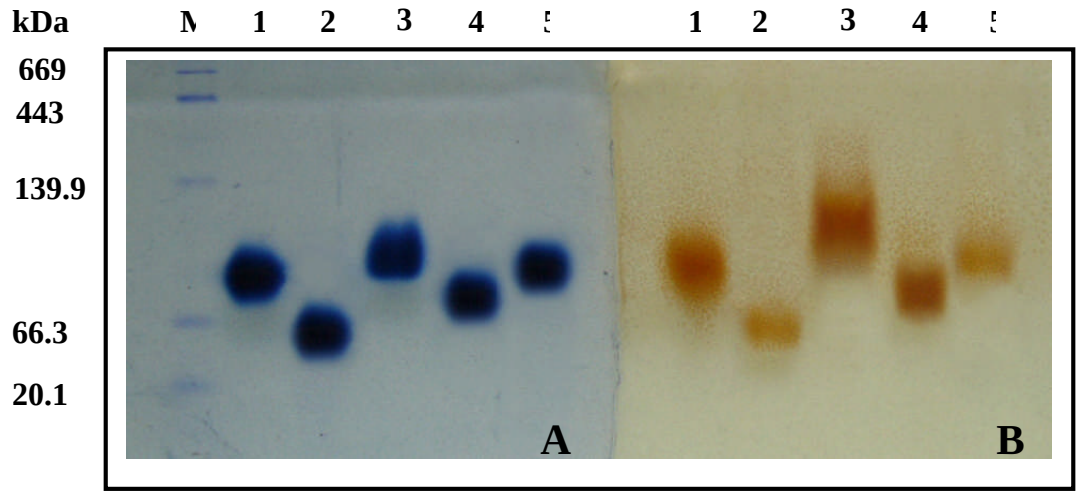

Figure 51 Non-denaturing native PAGE (10%) of laccase isozymes from *Ganoderma* sp. KU-Alk4. (A) Protein staining by Coomassie blue R-250 staining. Samples contained 6 µg (B) Activity staining with DMP. Samples contained 2 µg (B) protein. Lanes *1*: KULac 1, lanes *2*: KULac 2, lanes *3*: KULac 3, lane *4* KULac 5 and lane *5* KULac 4. Lanes *M*: Molecular weight markers were thyroglobulin, 669.0 kDa; ferritin, 443.0 kDa; lactate dehydrogenase, 139.9 kDa, albumin, 66.3 kDa and trypsin inhibitor, 20.1 kDa (DAIICHI Pure Chemicals).

Table 27 Molecular weight of laccase isozymes from *Ganoderma* sp. KU-Alk4.

Isozymes	Molecular weight (kDa)		Isoform
	SDS-PAGE	Native-PAGE	
KULac 1	67	89	Monomer
KULac 2	50	53	Monomer
KULac 3	71	112	Monomer
KULac 4	47	100	Dimer
KULac 5	65	74	Monomer

5.3.2 N-terminal amino acid sequence

Laccases are 520-550 amino acids in length, including a secrete signal sequence at N-terminal of 16-23 amino acids (Zhang *et al*., 2006). Fifteen amino acid sequences at the N-terminal of the 5 isozymes of *Ganoderma* sp. KU-Alk4 were determined and aligned to compare with the other laccases (Table 28). KULac 2 had the N-terminal sequence GIGPVADLTVRGGDI but KULac 1, 3, 4, and 5 had the identical N-terminal, GIGPVTDLTISNADI. When compared N-terminal amino acid sequence with that of the fungal genus, *Ganoderma lucidum*, the N-terminal sequence of 5 amino acid residues of laccase of *G. lucidum* was different from all KULacs by 1 amino acid (Ko *et al*., 2001).

When all 15 amino acid residues of KULac 2 were compared with the others previously reported, the sequence of KULac 2 showed the closest similarity to that of *Dichomitus squalens* but only by 67% (Périé *et al*., 1998), 60% similarity to laccase II and III of *T. versicolor* (Bourbonnais *et al*., 1995) and 53% similarity to the laccases of *T. villosa*, *Trichophyton rubrum* and *Pycnoporus cinnabarinus* (Yaver *et al*., 1996; Jung *et al*., 2002; Eggert *et al*., 1996).

The N-terminal sequences of KULac 1, 3, 4, and 5 showed the closest similarity, 93%, to the laccase of *D. squalens* (Périé *et al*., 1998). When compared with laccase II and III of *T. versicolor* and that of *Panus rudis* and *Coriolus hirsutus*, they were 67% similarity (Bourbonnais *et al*., 1995; Zhang *et al*., 2006; Kojima *et al*., 1990). There were 60% similarity to the laccases of *Ceriporiopsis subvermispora* and *Pycnoporus cinnabarinus* (Fukushima and Kirk, 1995; Eggert *et al*., 1996). Significant differences of the N-terminal sequences of all KULacs were from the laccases of *Phellinus ribis* and *Neurospora crassa*, with the similarities of only 13% (Min *et al*., 2001; Germann *et al*., 1988). *Ganoderma* sp. KU-Alk4 was closely related to *G. philippii* when its phylogenic comparison was done with ITS 4 (Part 6). From the different of amino acids sequence at N-terminal, it could be suggested that KULac 2 was a new laccase isozyme that could be produced by *Ganoderma*, KU-Alk4, in a certain stress of glucose.