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A new laccase from dried fruiting bodies of the monkey head mushroom *Hericium erinaceum*

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Abstract

A laccase with a novel N-terminal sequence, a molecular mass of 63 kDa, and inhibitory activity toward HIV-1 reverse transcriptase (IC_{50} = 9.5 μM) was isolated from dried fruiting bodies of the monkey head mushroom *Hericium erinaceum*. A chromatographic procedure involving ion exchange chromatography on DEAE–cellulose, CM–cellulose, and Q-Sepharose and fast protein liquid chromatography-gel filtration on Superdex 75 was employed. The laccase was adsorbed on DEAE–cellulose and Q-Sepharose but unadsorbed on CM–cellulose. High activity of the enzyme was observed at pH 3–5 and at 50–80 °C. Its activity was completely abolished at pH 8 and 9 and after boiling for 10 min. A temperature of 50 °C and a pH of 5.0 were optimal for its activity.

Keywords: Laccase; HIV; Mushroom

Mushrooms elaborate a variety of biomolecules including lectins [1,2], ribonucleases [3], ribosome inactivating proteins [4,5], and polysaccharopeptides.

From the fruiting bodies of the monkey head mushroom *Hericium erinaceum*, a sialic acid-binding lectin with a blocked N-terminus and a molecular mass of approximately 60 kDa has been isolated [6]. The monkey head mushroom also elaborates a polysaccharide with antitumor activity [7]. However, little else is known about its other biochemical constituents.

Laccases are a group of enzymes produced by ligninolytic mushrooms [8,9]. They find potential applications in textile dyes, biosensors, pulping, and detoxification of polluted water [10–15]. They have been isolated and/or the genes characterized from only some mushroom species despite the numerous mushroom species that exist [16–34]. The objective of the present investigation was to isolate and characterize a laccase from the monkey head mushroom which is a common edible mushroom in China.

Materials and methods

Isolation of laccase. A water extract of dried fruiting bodies (1.5 kg) of the monkey head mushroom *H. erinaceum* was prepared by homogenizing them in distilled water (3 ml/g) in a Waring blender. The homogenate was centrifuged and the supernatant was saved. Tris–HCl buffer (1 M, pH 7.4) was added to the supernatant until the concentration of Tris attained 10 mM. The supernatant was chromatographed on a 5 × 20 cm column of DEAE–cellulose (Sigma). The unadsorbed fraction (D1) eluted with 10 mM Tris–HCl buffer (pH 7.2) and devoid of laccase activity was discarded, while the adsorbed fraction (D2) with laccase activity was eluted with 0.5 M NaCl in the starting buffer. Fraction D2 was subsequently subjected to ion exchange chromatography on a CM–cellulose (Sigma) column (2.5 × 20 cm) in 10 mM NH_4OAc buffer (pH 4.5). Unadsorbed proteins (fraction CM1) were eluted with the same buffer while adsorbed proteins (fractions CM2 and CM3) were eluted with a linear NaCl gradient (0–1 M) in 10 mM NH_4OAc buffer (pH 4.5). Fraction CM1 containing laccase activity was next subjected to ion exchange chromatography on a Q-Sepharose (Amersham Biosciences) column (1.5 × 20 cm) in 10 mM NH_4OAc

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buffer (pH 9.2). After removal of unadsorbed proteins (fraction Q1) without laccase activity, adsorbed proteins (fractions Q2 and Q3) were eluted with a linear concentration (0–1 M) gradient of NaCl in the 10 mM NH_4HCO_3 buffer. Fraction Q2, which contained laccase activity, was fractionated on an FPLC-gel filtration Superdex 75 HR 10/30 column (Amersham Biosciences) in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (Amersham Biosciences). The first eluted peak (S1) represented purified laccase.

Assay for laccase activity. Laccase activity was assayed by measuring the oxidation of 2,7′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS) [18]. A modification of the method of Shin and Lee was used. An aliquot of enzyme solution was incubated in 1.3 ml of 67 mM sodium acetate buffer (pH 4.5) containing 1.54 mM ABTS at 30 °C. One unit of enzyme activity was defined as the amount of enzyme required to produce an absorbance increase at 405 nM of one per minute per milliliter of reaction mixture under the aforementioned condition.

Molecular mass determination by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by FPLC-gel filtration. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in accordance with the procedure of Laemmli and Favre [35], using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC-gel filtration was carried out using a Superdex 75 HR 10/30 column which had been calibrated with molecular mass standards (Amersham Biosciences).

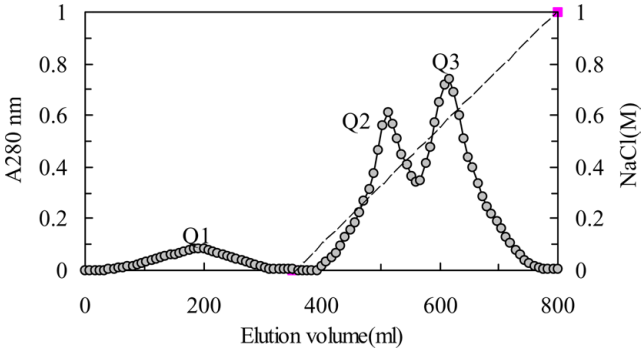

Fig. 1. Ion exchange chromatography on a Q-Sepharose column (1.5 × 20 cm). Sample: fraction of monkey head mushroom fruiting body extract adsorbed on DEAE–cellulose and subsequently unadsorbed on CM–cellulose. Binding buffer: 10 mM NH_4OAc buffer (pH 5.4). Buffer used to elute adsorbed fraction: 0–1 M NaCl in the binding buffer. The dotted line across the right half of the chromatogram represents the linear concentration gradient of NaCl.

Analysis of N-terminal amino acid sequence. Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP1000 HPLC system.

Assay for HIV-1 reverse transcriptase-inhibitory activity. The purified laccase was tested for this activity in view of the report that a mushroom laccase exhibited this activity [33].

The assay for HIV reverse transcriptase-inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer–Mannheim (Germany). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A) oligo(dT) 15. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecules, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity allows sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrates, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the isolated protein was calculated as percent inhibition as compared to a control without the protein [36,37].

Results

The fraction of the fruiting body extract of monkey head mushroom that was adsorbed on DEAE–cellulose in 10 mM Tris–HCl buffer (pH 7.4) and eluted with 0.5 M NaCl, and subsequently unadsorbed on CM–cellulose in 10 mM NH_4OAc buffer (pH 4.5) was fractionated on Q-Sepharose into a small unadsorbed peak Q1 and two much larger adsorbed peaks Q2 and Q3 (Fig. 1). Laccase activity was concentrated in fraction Q2 (Table 1). Fraction Q2 was resolved on Superdex 75 into two peaks S1 and S2 (Fig. 2). Laccase activity resided in S1 (Table 1). S1 exhibited a molecular mass of 63 kDa in SDS–PAGE (Fig. 3). The optimal temperature of the laccase was 50 °C. At 80 °C, 75% of the activity observed at 50 °C remained. The activity at 20 °C was about 65% of the activity at 50 °C (Fig. 4).

Table 1
Yields and laccase activities of aqueous extract and various chromatographic fractions (from 1.5 kg fresh fruiting body, activity assayed at 30 °C)

Chromatographic fractions	Protein yield (mg)	Laccase activity (U/mg)	Total activity (U)	Recovery of activity (%)	Purification fold
Extract	5340	0.8	4272	100	1
D1	1630	0.2	326	—	—
D2	2117	1.3	2752	64	1.6
CM1	936	2.0	1872	44	2.5
CM2	482	<0.1	<48	—	—
Q1	98.3	<0.1	<93	—	—
Q2	229.2	3.8	871	20	4.75
Q3	327.5	1.2	393	—	—
S1	44.7	11.9	532	12	15
S2	92.6	0.9	83	—	—

Laccase activity was determined at 30 °C.

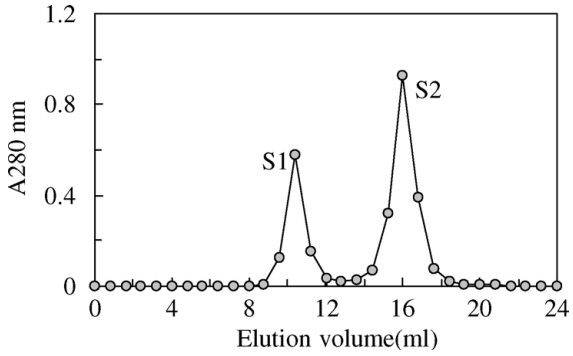

Fig. 2. Fast protein liquid chromatography-gel filtration on a Superdex 75 HR 10/30 column. Eluent: 0.2 M NH_4HCO_3 buffer (pH 8.5). Flow rate, 0.4 ml/min; fraction size, 0.8 ml.

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Left lane: molecular mass markers, from top downward, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). Right lane: fraction S1 (purified laccase).

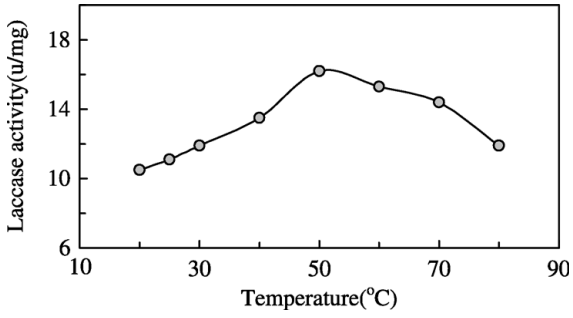

Fig. 4. Effect of temperature on laccase activity of *Hericium erinaceum* laccase.

The enzyme lost all of its activity after boiling for 10 min. The laccase activity did not vary dramatically over the pH range 3–5. The activity underwent an abrupt decline when the pH was raised to 6 and then to 7. No activity was discernible at pH 8 and 9 (Fig. 5). The laccase inhibited HIV-1 reverse transcriptase with an IC_{50} of 9.5 μM. The N-terminal sequence

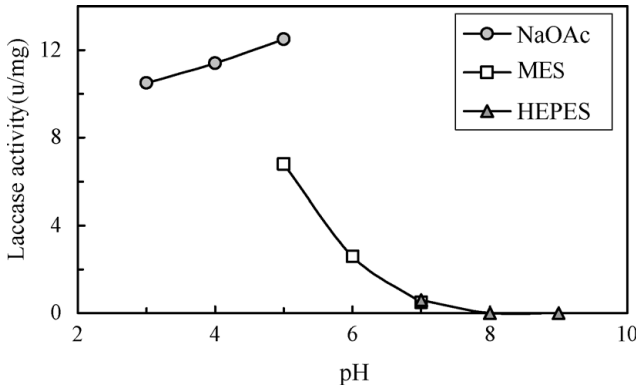

Fig. 5. Effect of pH on laccase activity of *Hericium erinaceum* laccase.

Table 2
N-terminal sequence comparison of laccases from *Hericium erinaceum* and other mushrooms

Hericium erinaceum laccase	AVDDDAEQ · IP
Agaricus bisporus laccase I	KTRTFDFDLVV
Agaricus bisporus laccase II	DTKTFNFDLVN
Basidiomycete PM1 laccase	SIGPVADLTIS
Cariporiopsis subvermispora laccase	AIGPVTDLEIT
Coriolus hirsutus laccase	GICTKANLVIT
Coriolus hirsutus laccase	AIGPTADLTIS
Phlebia radiata laccase	SIGPVTDFHII
Pleurotus eryngii laccase I	AXKKLDFHIII
Pleurotus eryngii laccase II	ATKKLDFHIII
Pycnoporus cinnabarinus laccase	AIGPVADLTLT
Trametes versicolor laccase I	AIGPVASLVVA
Trametes versicolor laccases II and III	GIGPVADLTIT

Identical corresponding amino acid residues are underlined.

of the laccase differed from those of other mushroom laccases (see Table 2).

Discussion

The molecular mass of *H. erinaceum* laccase falls within the range of molecular masses reported for other mushroom laccases [18,31]. The adsorption of *H. erinaceum* laccase on DEAE–cellulose is in line with the report that laccases from *Coriolus hirsutus* and *Rigidoporus lignosus* are adsorbed on DEAE–Sephacel [18,19], but in contrast to the inability of laccases from *Cantharellus cibarius*, *Tricholoma giganteum*, and *Albatrella dispansus* to bind to DEAE–cellulose [32–34]. Like *R. lignosus* laccase [18] which is adsorbed on Mono Q, *H. erinaceum* laccase is adsorbed on Q-Sepharose. In contrast to *C. hirsutus* laccase which is adsorbed on Hitrap SP and Mono S [18], and *T. giganteum* laccase which is adsorbed on CM–cellulose [33], *H. erinaceum* laccase is unadsorbed on CM–cellulose.

Laccases from *C. cibarius*, *T. giganteum*, and *A. dispansus* exhibit a pH optimum at pH 4 [32–34] compared to the optimal pH of 5 for *H. erinaceum* laccase. Like

laccases from *T. giganteum* and *A. dispansus* [32,33], *H. erinaceum* displays high activity at 50 °C and above. *C. cibarius* is less thermostable [34]. A pH of 3–4 and a temperature of 50 °C are required for *C. hirsutus* laccase to exhibit maximal activity [18]. *R. lignosus* laccase requires a temperature of 40 °C to exhibit maximal activity. It is less stable at pH values lower than pH 7. The high activity observed at 35–45 °C diminishes rapidly at 55–65 °C [19]. Thus, *H. erinaceum* laccase is closer to *C. hirsutus* laccase than *R. lignosus* laccase in pH and temperature optima although the former is much more thermostable.

The inhibitory effect of a mushroom (*T. giganteum*) laccase toward HIV-1 reverse transcriptase laccase has been demonstrated previously [33]. Its inhibitory potencies and that of *H. erinaceum* laccase are similar to those of other natural products [39]. However, laccases from *C. cibarius* and *A. dispansus* [32,34] lack similar activity. Some mushroom lectins [37] and ubiquitin-like proteins [38] also manifest HIV-1 reverse transcriptase-inhibitory effects. It is noteworthy that lectins and laccase are produced by both fruiting bodies [32–34,40,41] and mycelia [16–18,27,30,31,40] of mushrooms.

The N-terminal sequence of laccase shows little resemblance to those of published mushroom laccases [18] including those prepared in the authors' laboratory [32–34], indicating that it is a novel protein. From the preceding paragraphs it can be seen that different mushroom laccases may have different pH and temperature optima and chromatographic behavior on ion exchangers. *H. erinaceum* laccase has its own combination of characteristics.

References

[1] F. Yagi, M. Miyamoto, T. Abc, T. Minami, K. Tadera, I.J. Goldstein, Purification and carbohydrate-binding specificity of *Agrocybe cylindracea* lectin, Glycoconj. J. 14 (1997) 281–288.

[2] H.X. Wang, T.B. Ng, W.K. Liu, V.E.C. Ooi, S.T. Chang, Isolation and characterization of two distinct lectins with antiproliferative activity from the cultured mycelium of the edible mushroom *Tricholoma mongolicum*, Int. J. Pept. Protein Res. 46 (1995) 508–513.

[3] H. Kobayashi, N. Inokuchi, T. Koyama, H. Watenabe, M. Iwami, K. Ohgi, M. Irie, Primary structure of the nonspecific and adenylic acid preferential ribonuclease from the fruit bodies of *Lentinus edodes*, Biosci. Biotechnol. Biochem. 55 (1992) 2003–2010.

[4] H.X. Wang, T.B. Ng, Isolation and characterization of velutin, a novel low-molecular-weight ribosome-inactivating protein from winter mushroom (*Flammulina velutipes*) fruiting bodies, Life Sci. 68 (2001) 2151–2158.

[5] S.K. Lam, T.B. Ng, First simultaneous isolation of a ribosome inactivating protein and an antifungal protein from a mushroom (*Lyophyllum shimeiji*) together with evidence for synergism of their antifungal effects, Arch. Biochem. Biophys. 3939 (2001) 271–280.

[6] H. Kawagishi, H. Mori, A. Uno, A. Kimura, S. Chiba, A sialic acid-binding lectin from the mushroom *Hericium erinaceum*, FEBS Lett. 340 (1994) 56–58.

[7] T. Mizuno, T. Wasa, H. Itoh, C. Suzuki, H. Ukai, Antitumor-active polysaccharides isolated from the fruiting body of *Hericium erinaceum*, an edible and medicinal mushroom called yamabushi-take or houtou, Biosci. Biotechnol. Biochem. 56 (1992) 347–348.

[8] C.S. Evans, M.V. Dutton, F. Guillen, R.G. Veness, Enzymes and small molecular mass agents involved with lignocellulose degradation, FEMS Microbiol. Rev. 13 (1994) 235–240.

[9] C.F. Thurston, The structure and function of fungal laccases, Microbiology 140 (1994) 19–26.

[10] G. Palmieri, P. Giardina, L. Marzullo, B. Desiderio, G. Nitti, R. Cannio, G. Sannia. Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*, Appl. Microbiol. Biotechnol. 39 (1993) 632–636.

[11] O. Brenna, E. Bianchi, Immobilized laccase for phenolic removal in must and wine, Biotechnol. Lett. 16 (1994) 35–40.

[12] A.L. Ghindilis, A. Makower, F.F. Bier, F.W. Scheller, A laccase-glucose dehydrogenase recycling-enzyme electrode based on potentiometric mediatorless electrocatalytic detection, Anal. Methods Instrum. 2 (1995) 129–132.

[13] L. Martirani, P. Giardina, L. Marzullo, G. Sannia, Reduction of phenol content and toxicity in olive oil mill waste waters with the ligninolytic fungus *Pleurotus ostreatus*, Water Res. 30 (1996) 1914–1918.

[14] G. Palmieri, P. Giardina, B. Desiderio, L. Marzullo, M. Giamberini, G. Sannia, A new enzyme immobilization procedure using copper alginate gel: application to a fungal phenol oxidase, Enzyme Microbiol. Technol. 16 (1993) 151–158.

[15] I.D. Reid, M.G. Paice, Biological bleaching of kraft pulps by white-rot fungi and their enzymes, FEMS Microbiol. Rev. 13 (1994) 369–376.

[16] A. Leonowicz, M. Malinowska, Purification of the constitutive and inducible forms of laccase of the fungus *Pholiota mutabilis* by affinity chromatography, Acta Biochim. Pol. 29 (1982) 219–226.

[17] M.A. Pickard, D.W. Westlake, Purification, properties and substrate specificity of an inducible laccase from *Polyporus versicolor*, Can. J. Biochem. 48 (1970) 1351–1358.

[18] K.S. Shin, Y.J. Lee, Purification and characterization of a new member of the laccase family from the white-rot basidiomycete *Coriolus hirsutus*, Arch. Biochem. Biophys. 384 (2000) 109–115.

[19] M. Cambria, A. Cambria, S. Ragusa, E. Rizzarelli, Production, purification and properties of an extracellular laccase from *Rigidoporus lignosus*, Protein Express. Purif. 18 (2000) 141–147.

[20] T. Fukuda, H. Uchida, Y. Takashima, T. Uwajima, T. Kawabata, M. Suzuki, Degradation of bisphenol A by purified laccase from *Trametes villosa*, Biochem. Biophys. Res. Commun. 284 (2001) 704–706.

[21] J. Fernandez-Larrea, J. Stahl, Isolation and characterization of a laccase gene from *Podosporra arserina*, Mol. Gen. Genet. 252 (1996) 539–551.

[22] S. Chen, W. Ge, J.A. Buswell, Biochemical and molecular characterization of a laccase from the edible straw mushroom, *Volvariella volvacea*, Eur. J. Biochem. 271 (2004) 318–328.

[23] R.M. Berka, P. Schneider, E.J. Golightly, S.H. Brown, M. Madden, K.M. Brown, T. Halkier, K. Mondorf, F. Xu, Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oxyzae*, Appl. Environ. Microbiol. 63 (1997) 3151–3157.

[24] C. Eggert, P.R. La Fayette, U. Teng, K.E. Eriksson, J.F. Dean, Molecular analysis of a laccase gene from the white rot fungus *Pycnoporus cinnabarinus*, Appl. Environ. Microbiol. 64 (1998) 1766–1772.

[25] M. Schener, R. Fisher, Purification and characterization of laccase II of *Aspergillus nidulans*, Arch. Microbiol. 170 (1998) 78–84.

[26] P.M. Coll, J.M. Fernandez-Abalos, J.R. Villanueva, R. Santamaria, P. Perez, Purification and characterization of a peroxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971), Appl. Environ. Microbiol. 59 (1993) 2607–2613.
[27] C.R. Perry, S.E. Matcham, D.A. Ward, C.F. Thurston, The structure of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus*, J. Gen. Microbiol. 139 (Pt. 1) (1993) 171–179.
[28] M. Sakheimo, M.L. Niku-Paavola, J.K. Knowles, Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*, J. Gen. Microbiol. 137 (Pt. 7) (1991) 1531–1544.
[29] A. Sethuraman, D.E. Akin, K.E. Eriksson, Production of ligninolytic enzymes and synthetic lignin mineralization by the birds nest fungus *Cyathus stercoreus*, Appl. Microbiol. Biotechnol. 52 (1999) 689–697.
[30] R. Tinoco, M.A. Pickard, R. Vazquez-Duhalt, Kinetic differences of purified laccases from six *Pleurotus ostreatus* strains, Lett. Appl. Microbiol. 32 (2001) 331–335.
[31] A.M. Garzillo, M.C. Colao, V. Buonocore, R. Oliva, L. Falcigno, M. Saviano, A.M. Santoro, R. Zappala, R.P. Ronomo, C. Bianco, P. Giardina, G. Palmieri, G. Sannia, Structural and kinetic characterization of native laccases from *Pleurotus ostreatus*, *Rigidoporus lignosus* and *Trametes trogii*, J. Protein Chem. 20 (2001) 191–201.
[32] H.X. Wang, T.B. Ng, A novel laccase with fair thermostability from the edible wild mushroom (*Albatrella dispansus*), Biochem. Biophys. Res. Commun. 319 (2004) 381–385.
[33] H.X. Wang, T.B. Ng, Purification of a low-molecular-mass laccase with HIV reverse transcriptase inhibitory activity from the mushroom *Tricholoma giganteum*, Biochem. Biophys. Res. Commun. 315 (2004) 450–454.
[34] T.B. Ng, H.X. Wang, A homodimeric laccase with unique characteristics from the yellow mushroom *Cantharellus cibarius*, Biochem. Biophys. Res. Commun. 313 (2003) 37–41.
[35] U.K. Laemmli, M. Favre, Gel electrophoresis of proteins, J. Mol. Biol. 80 (1973) 575–599.
[36] R.A. Collins, T.B. Ng, W.P. Fong, C.C. Wan, H.W. Yeung, A comparison of human immunodeficiency virus type 1 inhibition by partially purified aqueous extracts of Chinese medicinal herbs, Life Sci. 60 (1997) 345–351.
[37] H.X. Wang, T.B. Ng, Examination of lectins, polysaccharopeptide, polysaccharide, alkaloid, coumarin and trypsin inhibitors for inhibitory activity against human immunodeficiency virus transcriptase and glycohydrolase, Planta Med. 67 (2001) 669–672.
[38] H.X. Wang, T.B. Ng, Isolation of a novel ubiquitin-like protein from *Pleurotus ostreatus* mushroom with anti-human immunodeficiency virus, translation-inhibitory and ribonuclease activities, Biochem. Biophys. Res. Commun. 276 (2000) 587–593.
[39] T.B. Ng, B. Huang, W.P. Fong, H.W. Yeung, Anti-HIV natural products with special emphasis on HIV-1 reverse transcriptase inhibitors, Life Sci. 61 (1997) 933–949.
[40] Q.B. She, T.B. Ng, W.K. Liu, A novel lectin with potent immunomodulatory activity isolated from both fruiting bodies and cultured mycelia of the edible mushroom *Volvariella volvacea*, Biochem. Biophys. Res. Commun. 247 (1998) 106–111.
[41] H.X. Wang, T.B. Ng, V.E.C. Ooi, Lectin activity in fruiting bodies of the edible mushroom *Tricholoma mongolicum*, Biochem. Mol. Biol. Int. 44 (1998) 135–141.