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SHORT COMMUNICATION

Biotransformation of mulberroside A from *Morus alba* results in enhancement of tyrosinase inhibition

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Abstract Mulberroside A, a glycosylated stilbene, was isolated and identified from the ethanol extract of the roots of *Morus alba*. Oxyresveratrol, the aglycone of mulberroside A, was produced by enzymatic hydrolysis of mulberroside A using the commercial enzyme Pectinex®. Mulberroside A and oxyresveratrol showed inhibitory activity against mushroom tyrosinase with an IC_{50} of 53.6 and 0.49 μM, respectively. The tyrosinase inhibitory activity of oxyresveratrol was thus approximately 110-fold higher than that of mulberroside A. Inhibition kinetics showed mulberroside A to be a competitive inhibitor of mushroom tyrosinase with L-tyrosine and L-DOPA as substrate. Oxyresveratrol showed mixed inhibition and noncompetitive inhibition against L-tyrosine and L-DOPA, respectively, as substrate. The results indicate that the tyrosinase inhibitory activity of mulberroside A was greatly enhanced by the bioconversion process.

Keywords Biotransformation · Mulberroside A · Oxyresveratrol · Tyrosinase inhibitor

Introduction

Mulberroside A is a glycosylated stilbene and one of the major components of the root extract of *Morus alba* [24]. In a previous study, mulberroside A showed hypocholesterolemic and antioxidant effects by inhibiting low-density lipoprotein (LDL) atherogenic modification and lipid peroxide formation [6]. Mulberroside A has been used as a raw material of skin-whitening cosmetics in Korea. Oxyresveratrol is an aglycone of mulberroside A and has various bioactivities. Oxyresveratrol showed strong tyrosinase inhibitory activity, and so may be suitable for use as a skin-whitening agent and as an anti-browning additive in food preparation [11, 14, 16, 29]. Oxyresveratrol has radical and reactive oxygen species (ROS)- and reactive nitrogen species (RNS)-scavenging properties, and has therefore been suggested as a potential protectant against ROS and RNS [17]. Oxyresveratrol exhibited neuroprotective effects in transient brain ischemia through antioxidative and antinitrosative activities, and prevented apoptotic cell death by limiting cytochrome c release and caspase-3 activation in mitochondria [1]. Another study reported anti-inflammatory activity of oxyresveratrol [5].

Chemical synthesis is a complex process and produces considerable toxic waste. Bioconversion is an eco-friendly and efficient method for enhancing the bioavailability of glycosylated stilbenes. Bioconversion of prodrugs enhances bioavailability [15], and bioconversion processes have

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been applied in various fields to improve the biological activity of bioactive molecules [2, 20, 25, 30]. Enzymatic conversion of glycosides into aglycones enhances their biological activities [18, 21, 22].

Mulberroside A and oxyresveratrol have shown inhibitory activity against tyrosinase. The tyrosinase inhibitory activity of mulberroside A may be improved by removing its glycoside moiety. The aim of the present work was to increase bioavailability of mulberroside A by biotransformation, and the bioconverted product was found to exert more potent tyrosinase inhibitory activity than did mulberroside A.

Materials and methods

Materials

The roots of *Morus alba* were harvested in Andong, Kyungsangbuk-do, Korea. Mushroom tyrosinase, L-tyrosine, 3,4-dihydroxyphenylalanine (L-DOPA), resveratrol, arbutin, and kojic acid were purchased from Sigma (St. Louis, MO, USA). Pectinex® was purchased from Novozymes (Denmark).

Preparation of mulberroside A and oxyresveratrol

The root of *M. alba* (3.6 kg) was extracted with ethanol (18 kg) at 80°C for 4 h and 15 kg of extract was collected. The extraction with residues was repeated with 15 kg of water at 80°C for 4 h and yielded 15 kg of second extract. The total extract was filtered through a diatomite filter (Filter press, SA 955) and the clean extract solution was collected. The extract was absorbed onto absorbent resin (SP 207, DIAION). The absorbent resin was sequentially washed with water (8 l) and methanol (6 l), and vacuum dried at 50°C for 30 h, yielding 80 g of crude extract powder. The crude extract powder was dissolved in methanol, and medium-pressure liquid chromatography (MPLC, FLASH 40 M, Biotage) was performed with a silica resin column (Silica gel si 60, WATCHERS). The mobile phase was chloroform and methanol (2.5:1), and the flow rate was 10 ml/min. Thin layer chromatography (TLC) fractionation (chloroform–methanol, 2.5:1) was performed and followed by vacuum drying, which yielded 4.8 g of mulberroside A. Purified mulberroside A (3.6 g) was dissolved in a small amount of methanol, the volume was then made up to 40 ml with water, and this was mixed with 4 ml of acetate buffer (pH 4.0) and 0.88 ml of Pectinex® and incubated at 60°C for 4 h with shaking at 120 rpm. The enzyme activity was stopped by heat treatment at 95°C for 2 min. The solution was absorbed onto absorbent resin (SP 207, DIAION). The absorbent resin was thoroughly washed with water, followed by washing with acetone, and vacuum dried at 50°C for 30 h. The powder was dissolved in methanol and MPLC (FLASH 40 M, Biotage) was performed with a silica resin column (Silica gel si 60, WATCHERS). The mobile phase was chloroform and methanol (2.5:1), and the flow rate was 10 ml/min. TLC fractionation (chloroform–methanol, 2.5:1) was performed and followed by vacuum drying, yielding 1.2 g of oxyresveratrol. The bioconversion process of mulberroside A to oxyresveratrol is shown in Fig. 1.

HPLC analysis and identification of purified compounds

The crude extract and TLC fraction were analyzed by high-performance liquid chromatography (HPLC, LCQ Advantage MAX, Thermo Electron, USA). The analysis was performed with Luna-Pak® C-18 (5 μm, 4.6 mm i.d. × 250 mm). The mobile phase was acetonitrile (CH_3CN) and 10 mM phosphoric acid (H_3PO_4) (35:65). Flow rate was 1.0 ml/min and the effluent was monitored at 325 nm with a Photodiode Array (PDA) detector. Identification of the purified mulberroside A and oxyresveratrol was carried out by ^1H NMR (Varian 400-MR, 500 MHz, Varian Inc., USA), ^{13}C NMR, and MALDI-Mass (Voyager-DE STR Biospectrometry Workstation, Applied Biosystems Inc., USA). The purity was calculated by HPLC with resveratrol as standard. The purity of mulberroside A and oxyresveratrol was over 95 and 98%, respectively.

Mushroom tyrosinase inhibition assay

Assays were performed as previously described with slight modifications [13]. Briefly, reaction mixtures, consisting of 150 μl of 0.1 M phosphate buffer (pH 6.8), 10 μl of mushroom tyrosinase (2,000 U/ml), 10 μl of a sample in dimethyl sulfoxide (DMSO), and 100 μl of distilled water, were mixed in a 96-well microplate and pre-incubated at 25°C for 10 min, and then 30 μl of 2 mM L-tyrosine or L-DOPA was added. Optical densities (OD) were measured at 475 nm after incubation at 25°C for 30 min using a Spectra max 340pc microplate reader (Molecular Devices). Tyrosinase inhibition percentage was calculated as follows: $\%\ \text{inhibition} = \{[(A - B) - (C - D)]/(A - B)\} \times 100$ (A = OD without the test substance but with tyrosinase; B = OD without the test substance and without tyrosinase; C = OD with the test substance and with tyrosinase; D = OD with the test substance but without tyrosinase; all ODs were determined at 475 nm). Inhibitor constants (Ki) in the presence of mulberroside A and oxyresveratrol against mushroom tyrosinase were determined by

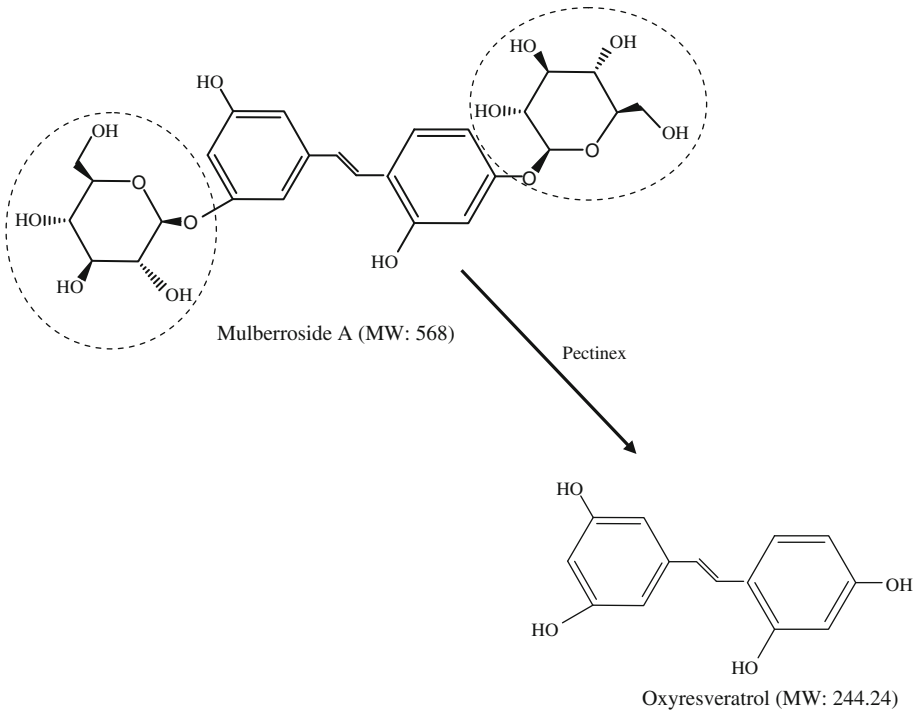

Fig. 1 Bioconversion of mulberroside A into oxyresveratrol

Lineweaver–Burk's plot using various concentrations of L-tyrosine or L-DOPA as substrate.

Results and discussion

Mulberroside A was purified from the roots of *M. alba,* and the purity and structure of mulberroside A was confirmed by HPLC, NMR, and mass spectral data. To obtain oxyresveratrol more easily and improve the inhibitory activity against tyrosinase, we performed a bioconversion of mulberroside A with the commercial enzyme Pectinex®, which contains β-glucosidase activity. The purified mulberroside A was treated with Pectinex® to remove the glycosylated moiety of mulberroside A. The enzyme-hydrolyzed material was purified and the purified compound was confirmed as a homogeneous compound by HPLC analysis, which showed a single symmetrical peak with a retention time of 4.5 min on a Luna-Pak C18 column. The compound was identified as oxyresveratrol by ^{1}H-NMR, ^{13}C-NMR, and MALDI-Mass. A total of 4.8 g of mulberroside A was yielded from the ethanol extract of the roots of *M. alba* (3.6 kg). Treatment with Pectinex® resulted in about 80% of the mulberroside A being converted into oxyresveratrol with a purity of over 98%. The oxyresveratrol, a yellow powder, showed a molecular weight of 243 and its detailed data, HPLC profile, ^{1}H-NMR, ^{13}C-NMR, and MALDI-Mass spectra are available in the supplementary material.

Mulberroside A and oxyresveratrol are relatively insoluble in water, and therefore they were dissolved in DMSO. For comparative purposes, the tyrosinase inhibitory activity was assayed against kojic acid and arbutin as positive standards, along with mulberroside A and oxyresveratrol. Oxyresveratrol showed strong inhibitory activity against mushroom tyrosinase (Fig. 2). Tyrosinase was almost completely inhibited at about 2.5 μM oxyresveratrol, while mulberroside A and kojic acid completely inhibited the enzyme activity at over 500 and 250 μM, respectively. Arbutin showed poor inhibitory activity against the tyrosinase with 85.5% inhibition at 3,000 μM.

The inhibitory dose required to reduce tyrosinase activity to 50% (IC_{50}) was determined by adding mulberroside A, oxyresveratrol, kojic acid, or arbutin to an assay system containing L-tyrosine or L-DOPA as a substrate of mushroom tyrosinase, and their respective IC_{50} values are shown in Table 1. With L-tyrosine as the tyrosinase substrate, oxyresveratrol showed 110-fold higher tyrosinase inhibitory activity than did mulberroside A, and 43-fold and 1,503-fold higher inhibitory activity than kojic acid and arbutin, respectively. The tyrosinase inhibitory activity of oxyresveratrol against L-DOPA as a substrate was 33-fold higher than that of kojic acid. On the other hand, mulberroside A and arbutin did not show an inhibitory effect at concentrations of up to 1,000 and 3,000 μM, respectively. The IC_{50} values of oxyresveratrol and kojic acid with L-tyrosine as a substrate were lower than those with L-DOPA as a substrate. Thus, the inhibitory effects of

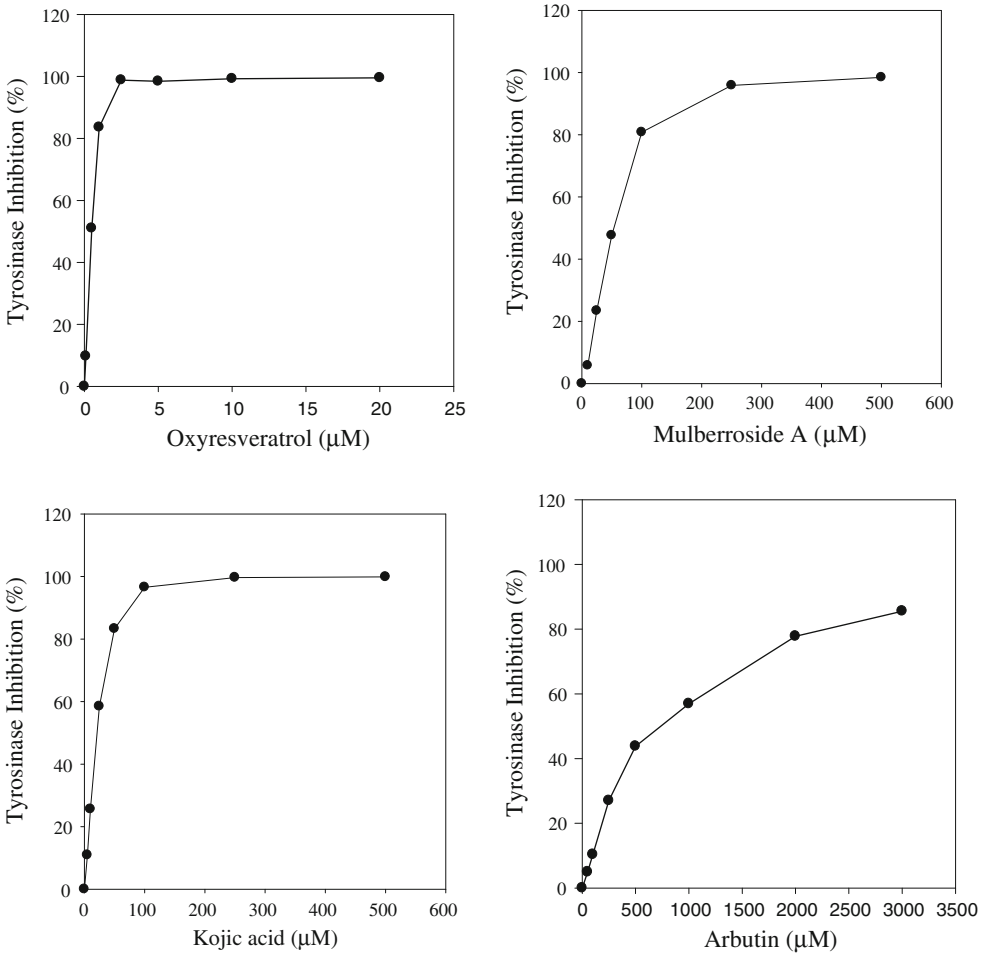

Fig. 2 Mushroom tyrosinase inhibition, with L-tyrosine as a substrate, exerted by oxyresveratrol, mulberroside A, kojic acid, and arbutin

Table 1 Inhibitory effects on mushroom tyrosinase by mulberroside A, oxyresveratrol, kojic acid, and arbutin

Compound	IC_{50} (μM)	
	L-Tyrosine	L-DOPA
Arbutin	736.5 ± 9.2	–[a]
Kojic acid	21.1 ± 3.1	387.9 ± 10.2
Mulberroside A	53.6 ± 2.3	–
Oxyresveratrol	0.49 ± 0.06	11.9 ± 1.2

[a] No inhibition

oxyresveratrol and kojic acid on the oxidation of L-DOPA were less than their inhibitory effects on the hydroxylation of L-tyrosine.

Inhibition kinetics, as determined using a Lineweaver–Burk plot, showed mulberroside A to be a competitive inhibitor of mushroom tyrosinase with a *Ki* value of 49.821 μM against L-tyrosine and 124.764 μM against L-DOPA (Fig. 3, Table 2). Oxyresveratrol was a mixed-type inhibitor of mushroom tyrosinase against L-tyrosine with *Ki* and *Ki'* values of 1.093 and 0.521 μM, respectively, and a noncompetitive inhibitor of the enzyme against L-DOPA with a *Ki* value of 1.272 μM (Fig. 4, Table 2). The *Ki* values of oxyresveratrol were lower than those of mulberroside A, consistent with the results of IC_{50} values.

Biotransformation has been conducted to improve bioavailability of flavonoids [7, 8, 23]. Flavonoids generally contain glycosides; however, flavonoids with glycosides removed have shown improved antioxidant, anticancer, and antimicrobial activities and have also more effectively prevented bone loss. Flavonoid aglycones exhibit greater bioactivity than their glycosides [18, 21, 22]. Resveratrol, an aglycone of piceid, was more active than its glycoside in inhibition of tyrosinase [10], and showed 6.9- and 8.3-fold higher activity for DOPA and tyrosine oxidation, respectively, than those of piceid. The antioxidant activity and anti-inflammatory effects of mulberroside A and oxyresveratrol were measured and both showed inhibitory effects against $FeSO_4/H_2O_2$-induced lipid peroxidation and carrageenin-induced paw edema [5]. IC_{50} values of the antioxidative activity of mulberroside A and oxyresveratrol against lipid peroxidation were 78.4 and 3.6 μM, respectively. The anti-inflammatory effects of oxyresveratrol

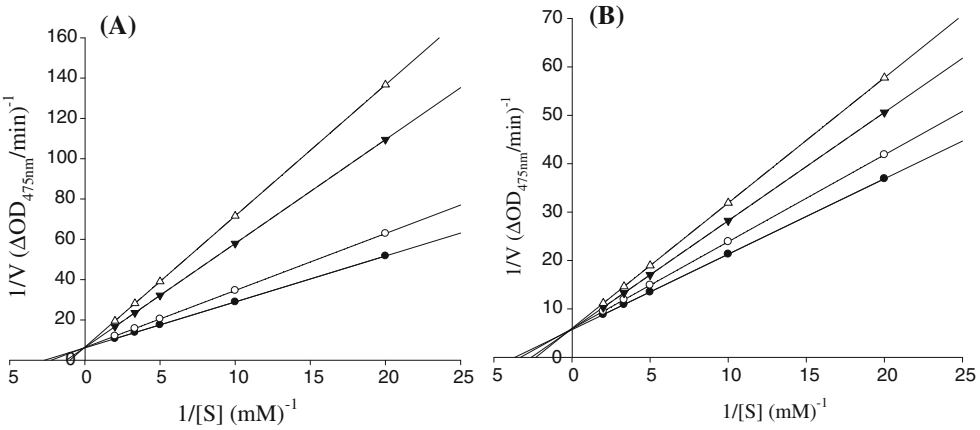

Fig. 3 Lineweaver-Burk plots of mushroom tyrosinase and L-tyrosine (**a**) or L-DOPA (**b**) with mulberroside A. The *symbols* represent without (*filled circle*) and with (*open circle*) 10 μM, (*filled triangle*) 50 μM, and (*open triangle*) 100 μM mulberroside A

Table 2 K_i values for the monophenolase and diphenolase activities of mushroom tyrosinase in the presence of oxyresveratrol and mulberroside A

Inhibitory agent	Substrate	*Ki* (μM)	Mode of inhibition
Oxyresveratrol	L-Tyrosine	1.093 ± 0.052 (*Ki*)[a]	Mixed[b]
		0.521 ± 0.172 (*Ki'*)	
	L-DOPA	1.272 ± 0.026	Noncompetitive
Mulberroside A	L-Tyrosine	49.821 ± 0.637	Competitive
	L-DOPA	124.764 ± 1.771	Competitive

[a] $Ki = [E][I]/[EI]$, $Ki' = [ES][I]/[ESI]$

[b] Competitive-noncompetitive

were much greater than those of mulberroside A. The results of the present study also show enhanced bioactivity of a flavonoid compound through biotransformation.

Oxyresveratrol has been chemically synthesized [4] or directly purified from the extracts of various herb plants [14, 17, 26]. A study reported that mulberroside A was hydrolyzed by *β*-glucosidase and converted into the aglycone, oxyresveratrol [9], and identified mulberroside A as an oxyresveratrol-di-*O*-glucoside. Oxyresveratrol exhibited strong antioxidant activity, inhibitory activity for tyrosinase, and antibrowning effects [14, 17]. A previous study showed that at a concentration of greater than 10 μM, oxyresveratrol purified from *M. alba* showed over 90% of inhibition of the DOPA oxidase activity of mushroom tyrosinase, exerting approximately 150-fold greater inhibitory effects on the enzyme activity than did resveratrol [29]. Piceid is a glycoside of hydroxystilbene and showed poor inhibitory activity up to a concentration of 500 μM. Comparing to resveratrol and piceid, the number and position of hydroxyl groups in oxyresveratrol seem to play a critical role in the inhibitory effect on the DOPA oxidase activity of mushroom tyrosinase. An enzyme where the catalytic site includes a proton acceptor may be inhibited by increasing the hydrogen ion concentration. A three-dimensional model of mammalian tyrosinase revealed that the active site of the enzyme contains hydrogen acceptors for the NH-groups of the imidazole rings of the copper-binding histidines [27]. The bulky glycosyl moiety may also affect enzyme activity through steric hindrance. In line with this, we obtained similar results. Kojic acid has two hydroxyl groups, arbutin has a hydroxyl group and a glycosyl moiety, and mulberroside A has two hydroxyl groups and two glycosyl groups. The inhibitory effects of oxyresveratrol on tyrosine oxidase and DOPA oxidase activities of mushroom tyrosinase were much higher than those of kojic acid, arbutin, and mulberroside A. The results obtained in the present study support the previous conclusions. The phenolic structure, however, might not exert an inhibitory effect on the enzyme activity, because the inhibitory activity of kojic acid with L-tyrosine as a substrate was two-fold more and 43-fold less potent than those of mulberroside A and oxyresveratrol, respectively. The previous report showed that flavonoids and stilbenes having the 4-susbstituted resorcinol skeleton such as oxyresveratrol and 4-ethylresorcinol have potent tyrosinase inhibitory activity [28]. However, a 4-susbstituted resorcinol skeleton was not the only factor to show tyrosinase inhibitory activity in flavonoids. Although artocarpin having a 4-susbstituted resorcinol skeleton, it showed decreased inhibitory activity probably because of its steric hindrance with a bulky C3 substituent. Mulberroside A has more bulky structure than oxyresveratrol because of a glycosyl group of mulberroside A. This might decrease the inhibitory activity of mulberroside A compared to oxyresveratrol against mushroom tyrosinase. The report also showed that hydrophobic as well as less bulky substituents were important for the tyrosinase inhibitory activity [28].

Kojic acid inhibited mushroom tyrosinase with a competitive inhibition mode and a mixed inhibition mode with L-tyrosine and DL-DOPA, respectively, as substrate [3]. The *Ki* values of kojic acid were 30 and 20 μM against monophenolase and diphenolase, respectively. The results obtained in this study showed that oxyresveratrol more effectively inhibited the enzyme than did a well-known

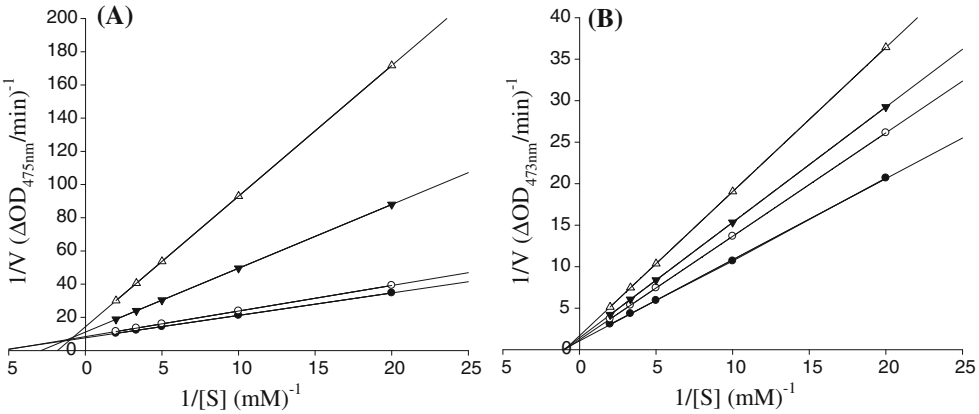

Fig. 4 Lineweaver-Burk plots of mushroom tyrosinase and L-tyrosine (**a**) or L-DOPA (**b**) with oxyresveratrol. The *symbols* represent without (*filled circle*) and with (*open circle*) 0.1 μM, (*filled triangle*) 0.5 μM, and (*open triangle*) 1.0 μM oxyresveratrol

inhibitor, kojic acid. Oxyresveratrol previously showed noncompetitive inhibition of the DOPA oxidase activity of mushroom tyrosinase with a *Ki* value of 0.91 μM [29]. Oxyresveratrol was a reversible noncompetitive inhibitor of mushroom tyrosinase with the same *Ki* and *Ki'* values of 0.32–0.43 μM with L-tyrosine as a substrate [11]. In this study, oxyresveratrol showed mixed and noncompetitive inhibition of tyrosinase with L-tyrosine and L-DOPA, respectively, as substrate. The *Ki* value of 1.272 against DOPA oxidase was similar to that in the previous report. In the mixed inhibition by oxyresveratrol on mushroom tyrosinase with L-tyrosine as substrate, the *Ki* value was higher than the *Ki'*, indicating competitive-noncompetitive inhibition. On the other hand, mulberroside A was a competitive inhibitor of the enzyme with L-tyrosine and L-DOPA as substrate. Tyrosinase catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) [12, 19]. Considering the IC_{50} and *Ki* values, oxyresveratrol exhibited greater influence on the monophenolase activity of mushroom tyrosinase than on the diphenolase activity of the enzyme.

Conclusions

Bioconversion will contribute to the replacement of the current expensive and environmentally unfriendly multi-step chemical conversion process for producing oxyresveratrol. Active compounds from the ethanol extract of *M. alba* root contained an inhibitory agent of tyrosinase, which was identified as mulberroside A, and oxyresveratrol was produced from mulberroside A by treatment with Pectinex®, which contains *β*-glucosidase. Compared to mulberroside A, oxyresveratrol showed highly enhanced inhibitory activity against tyrosinase. In conclusion, deglycosylation of mulberroside A by biotransformation greatly improves the tyrosinase inhibitory activity.

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