

Hen egg white lysozyme as an inhibitor of mushroom tyrosinase

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Abstract We report a kinetics study on hen egg white lysozyme's (HEWL) inhibitory effect on mushroom tyrosinase catalysis of 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) or L-tyrosine. For the first time, we demonstrate HEWL as a robust inhibitor against mushroom tyrosinase in catalysis of both substrates. The kinetics pattern matches a mixed (mostly non-competitive) partial inhibition. K_i and ID_{50} value of HEWL are more than 20-fold lower than that of kojic acid, a well-known chemical inhibitor of mushroom tyrosinase. K_i , α value and β value, are almost identical in both experiments (L-DOPA and L-tyrosine as substrates, respectively), which suggests this common inhibition mechanism affects both steps. The inhibitory effect increases as both proteins were mixed and pre-incubated for less than 1 h. HEWL-depletion only removed about half of the inhibitory effect. Here we propose a novel function of HEWL, which combines the reversible inhibition and the irreversible inactivation toward mushroom tyrosinase. Discovery of HEWL as an inhibitor to mushroom tyrosinase catalysis may be commercially valuable in the food, medical and cosmetic industries.

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1. Introduction

In our continuing investigation of mosquito innate immunity, we studied melanization of abiotic targets [1]. One type of target, Sephadex CM beads was melanized in the L3-5 strain of *Anopheles gambiae* but not in the 4a rr strain. Beads removed from the 4a rr were not melanized when transferred to L3-5 [2]. One protein on the beads was lysozyme. Pre-incubation of beads in HEWL (hen egg white lysozyme) nearly eliminated melanization in L3-5 strain. Thus, lysozyme was identified as a potential melanin production inhibitor.

Lysozymes (peptidoglycan *N*-acetylmuramoylhydrolase, EC 3.2.1.17) are proteins that have been defined by their muramidase activity, meaning the ability to cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetyl glucosamine (Glc-

NAc) in the peptidoglycan layer of bacterial cell walls. Lysozyme has been reported to have antibacterial [3], antifungal [4] and antiviral [5] activities, but a biological role in regulating melanin production is not known.

Tyrosinase (monophenol, dihydroxy-L-phenylalanin: oxygen oxidoreductase, EC 1.14.18.1) is a copper-dependent, multi-functional enzyme that catalyzes two different reactions: the hydroxylation of L-tyrosine to 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) (monophenolase activity) followed by the oxidation of L-DOPA to dopaquinone (diphenolase activity) [6]. Tyrosinase has important functions in insect biology, as well as economic importance in the food and cosmetic industries. In insects, several roles of this enzyme have been reported for pigmentation, wound healing, parasite encapsulation, and sclerotization [7–11]. In the food industry, tyrosinase is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing [12–14]. In addition, tyrosinase inhibitors are becoming important constituents of cosmetic products that relate to hyperpigmentation [15]. Because of these important aspects, there is a concerted effort to search for naturally occurring tyrosinase inhibitors [16,17].

To study in depth tyrosinase inhibition by lysozyme, we studied the effect of HEWL on the monophenolase and diphenolase activities of mushroom tyrosinase, and determined the ID_{50} and K_i .

2. Results

2.1. Inhibitory effect of HEWL against mushroom tyrosinase activity

To study tyrosinase inhibition by lysozyme, we examined HEWL's effect on the diphenolase and monophenolase activities of mushroom tyrosinase, and determined ID_{50} and K_i . ID_{50} is the required concentration of HEWL to reduce the mushroom tyrosinase activity to 50% of that in a control reaction containing no inhibitor under the same experimental conditions. K_i is an enzyme/inhibitor dissociation constant.

When the diphenolase activity of mushroom tyrosinase was examined by using L-DOPA as substrate, the reaction immediately reached a steady-state rate (Fig. 1, curve A). The presence of HEWL in the assay medium resulted in reduction in the diphenolase activity (Fig. 1, curve B). In addition, mushroom tyrosinase also shows monophenolase activity. When the enzymatic reaction was started by the addition of on L-tyrosine, a marked lag period, characteristic of monophenolase activity [18], was observed, simultaneously with the appearance of dopachrome (Fig. 1, curve C). The system reached a constant rate after the lag period. As can be seen from curves C and D

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Abbreviations: HEWL, hen egg white lysozyme; L-DOPA, 3-(3,4-dihydroxyphenyl)-L-alanine

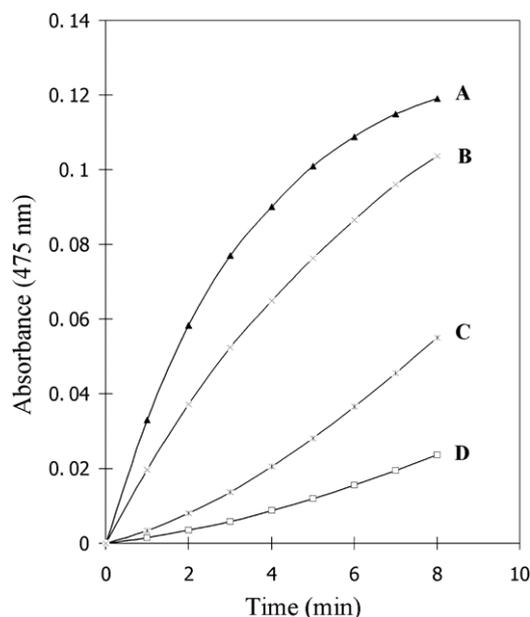


Fig. 1. Progress curves for the inhibition of mushroom tyrosinase by HEWL. Curves A and B show the effect of the HEWL on the diphenolase activity of the enzyme. Curves C and D show the effect of HEWL on the monophenolase activity of the enzyme. The reaction media contained L-DOPA (0.1 mM) or L-tyrosine (0.4 mM) in a final concentration of 30 mM MOPS buffer, pH 6.5. HEWL concentrations were (A, C) = 0, B = 0.8 μ M, D = 0.48 μ M. The reaction was started by the addition of the substrate.

in Fig. 1, HEWL also slowed the rate of dopachrome formation when L-tyrosine was used as substrate, behaving, therefore, as an inhibitor of the monophenolase activity of mushroom tyrosinase.

To obtain further information on the type of inhibition exerted by HEWL on mushroom tyrosinase, diphenolase activities (V) were measured (Fig. 2). The kinetics behavior of HEWL during the oxidation of L-DOPA was studied (Fig. 2A and D). Kinetics data from Dixon plots showed partial mixed inhibition towards mushroom tyrosinase catalysis of L-DOPA (Fig. 2D). The K_i and ID_{50} values estimated were 0.075 and 0.32 μ M.

To ascertain whether HEWL also behaved as a partial mixed inhibitor as regards the monophenolase activity of mushroom tyrosinase, the rate of dopachrome accumulation was measured as a function of L-tyrosine concentration for several concentrations of inhibitor. The Dixon plot obtained (Fig. 2E) shows that HEWL is also a partial mixed inhibitor of the monophenolase activity of mushroom tyrosinase. The K_i and ID_{50} value obtained for this plot were 0.12 and 0.20 μ M. Mushroom tyrosinase inhibitory effects by HEWL and kojic acid are presented in Table 1. Kojic acid showed non-competitive inhibition with ID_{50} of 4.0 μ M (Fig. 2C). HEWL exhibited 20-fold lower ID_{50} for mushroom tyrosinase than the positive control, kojic acid.

2.2. Pre-incubation of HEWL with mushroom tyrosinase

The mushroom tyrosinase inhibitory activity by HEWL was influenced by pre-incubation of HEWL with the tyrosinase. When HEWL (0.5 μ M) and mushroom tyrosinase (7.5 U) were mixed just before measurement, the inhibition was ca. 7.93%.

This value increased by almost 4-fold (ca. 30.25%) when mushroom tyrosinase was mixed and incubated with HEWL at 25 °C for 1 h. After 1 h, this value drops gradually as demonstrated in Fig. 3.

2.3. HEWL depletion experiment

A significant decrease in diphenolase activity of mushroom tyrosinase was observed after mushroom tyrosinase and HEWL were mixed and incubated without substrate (L-DOPA). This showed that HEWL directly inactivates the mushroom tyrosinase diphenolase activity. To examine whether this inhibition effect by HEWL is reversible, we performed a HEWL-depletion experiment to measure the non-recoverable inhibition effect. First, controls were performed. Two samples of mushroom tyrosinase were incubated at 25 °C for 1 h, only one of which was followed by incubation with Sephadex CM beads. As expected, controls of mushroom tyrosinase, with or without HEWL-depletion procedure, have almost identical amount mushroom tyrosinase (Fig. 4B, lanes 1 and 2) and diphenolase activity (Fig. 4A, \blacktriangle and \triangle). Consistently, no mushroom tyrosinase was detected in elutes from the corresponding Sephadex CM beads (Fig. 4B, lane 3).

When mushroom tyrosinase was incubated with HEWL for 1 h at 25 °C (Fig. 4B, lane 4), 35.7% of diphenolase activity was suppressed (Fig. 4A, \blacksquare). Sephadex CM bead depletion resulted in loss of detectable HEWL (Fig. 4B, lane 5) while no mushroom tyrosinase was consumed by this procedure (Fig. 4B, lane 6). After the HEWL-depletion procedure, this sample exhibited a recovery of 15.7% of diphenolase activity (Fig. 4A, +). In other words, about half of the inhibitory effect was recovered/reversed by HEWL-depletion.

3. Discussion

Recently, a novel function of mosquito lysozyme has been found in our laboratory. *A. gambiae* lysozyme shows an anti-melanization effect in mosquitoes (unpublished). HEWL was also found to inhibit melanin formation in vivo in *A. gambiae*. We observed in vitro inhibitory effect of HEWL to both mushroom tyrosinase and mosquito tyrosinase (Li et al., unpublished). To explore the biochemical mechanism of this antimelanization effect, we characterized in vitro an interaction between HEWL and mushroom tyrosinase. For convenience, we chose commercially available HEWL and mushroom tyrosinase as models.

The kinetics of HEWL's inhibitory effect on mushroom tyrosinase has been measured during catalysis of both L-DOPA and L-tyrosine. HEWL exhibits a robust inhibitory activity against mushroom tyrosinase. K_i and ID_{50} value of HEWL are more than 20-fold lower than that of kojic acid, a well-known inhibitor of mushroom tyrosinase [19]. However, due to the higher molecular weight of HEWL (14 kDa), their inhibition efficiency by weight is in the same range. This low K_i and ID_{50} are compatible with the physiological environment of insects [20–22], which suggests that lysozyme may be involved in the physiological control of melanin production in these organisms.

The kinetic pattern of HEWL inhibition of mushroom tyrosinase matches a mixed (almost non-competitive) partial inhibition. This mixed-type inhibition can arise in many ways [23].

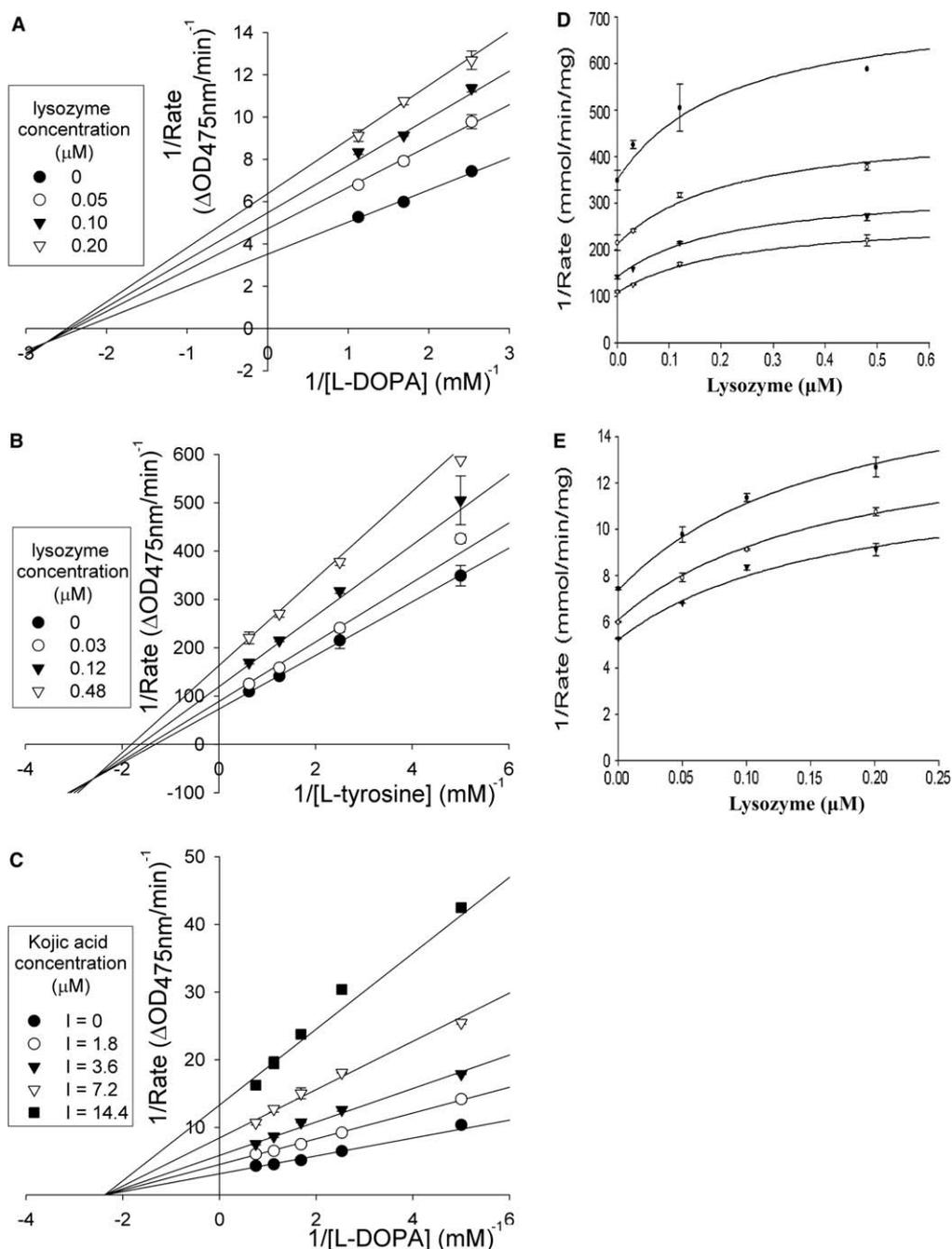


Fig. 2. Lineweaver–Burk (A, B, C) and Dixon (D, E) plots of mushroom tyrosinase: (A, D) With L-DOPA as a substrate, in the presence of HEWL. (B, E) With L-tyrosine as a substrate, in the presence of HEWL. (C) With L-DOPA as a substrate, in the presence of kojic acid. The reaction mixture contained the indicated concentration of substrate and 7.5 U of mushroom tyrosinase in a total volume of 250 μl of 30 mM MOPS buffer (pH 6.5). The final concentrations of the inhibitors are indicated on the left side of each Lineweaver–Burk plot. Data were obtained as mean values of $1/V$ of three independent tests. $1/V$ is defined as the inverse of the increase of absorbance at a wave-length of 475 nm per min.

HEWL may bind to mushroom tyrosinase and induce a conformational change. This conformational change could reduce mushroom tyrosinase activity. Alternatively, HEWL might cause an irreversible modification of mushroom tyrosinase through its enzyme activity. Finally, the inhibition could arise because HEWL interacts with a later intermediate in the reaction but not with the initial enzyme–substrate complex [19,23].

Inhibition activity increased during the first hour of pre-incubation but started to decrease afterwards as demonstrated

in Fig. 3. The dependence of inhibition on pre-incubation time excluded the possibility that the inhibitor interacts with either intermediate or substrate–enzyme complex. In addition, the slow decay of lysozyme inhibitory effect suggested tyrosinase activity was restored when lysozyme started to lose its activity. Lysozyme is likely to bind to mushroom tyrosinase and induce a conformational change.

To confirm whether HEWL's inhibitory effect is reversible, we performed a comparative experiment. In this experiment,

Table 1
Inhibitory effect of HEWL and kojic acid on mushroom tyrosinase activity (substrate: L-DOPA and L-tyrosine)

Figure	A	B	C
Inhibitor	HEWL	HEWL	Kojic acid
Substrate	L-DOPA (0.4 mM)	L-tyrosine (0.8 mM)	L-DOPA (0.4 mM)
K_i (μM)	0.075 ± 0.015	0.11 ± 0.02	4.0 ± 0.2
ID_{50} (μM)	0.32 ± 0.06	0.20 ± 0.04	4.0 ± 0.2
Inhibitory type	Partial mixed	Partial mixed	Partial non-competitive
α	0.91 ± 0.2	0.68 ± 0.1	N/A
β	0.40 ± 0.05	0.36 ± 0.03	0.03 ± 0.02
V_{max}	0.28 ± 0.01	0.014 ± 0.004	0.32 ± 0.06
K_m (mM)	0.43 ± 0.04	0.76 ± 0.05	0.42 ± 0.02

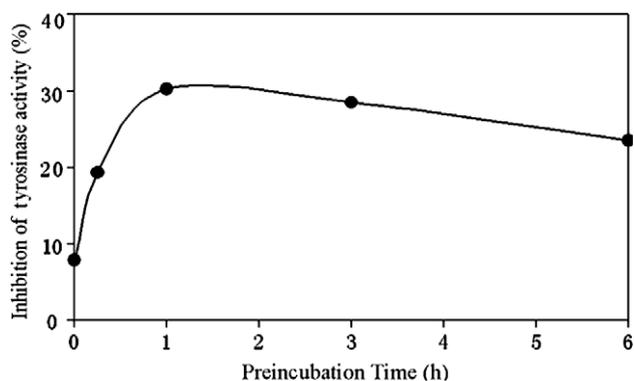


Fig. 3. Inhibitory effects of HEWL on mushroom tyrosinase activity with several pre-incubation times. HEWL (75 μl , 0.25 μM) was added to mushroom tyrosinase (75 μl , 7.5 U) and the mixture was incubated at 25 $^{\circ}\text{C}$ for different times (0 min, 15 min, 1 h, 3 h and 6 h). The reaction was started by addition of the 2 mM L-DOPA to the mixture. Mushroom tyrosinase diphenolase activity was determined as the increase of absorbance at 475 nm. Values are calculated from three independent tests.

we were able to totally deplete HEWL from a mixture of pre-incubated HEWL-tyrosinase by Sephadex CM bead affinity. In comparison with the control, only half of the mushroom

tyrosinase activity was recovered in HEWL-depleted sample (Fig. 4A). The other half of mushroom tyrosinase activity was not restored after HEWL depletion and one hour pre-incubation, which can be explained by 2 models. The first model we hypothesize is a mixed inhibition mechanism. In this model, HEWL binds to glycosidic linkage in tyrosinase and induce its conformational change. Some of the bound HEWL continues to non-specifically cleave glycosidic linkages and induces irreversible inhibition. After depletion of HEWL, only the portion of reversibly-inhibited tyrosinase is restored as to its diphenolase activity. The other potential model is a slow reversible inhibition mechanism. In this model, all tyrosinase is inhibited reversibly due to slow conformational change. It took 1 h for HEWL to bind to tyrosinase (not necessary to the glycosidic linkage). After depletion, dissociated tyrosinase begins to change back to its active conformation slowly (about half amount changed in one hour). Both models consist of reversible inhibition which is supported by the experiment in Fig. 3. Note that the inhibition effect drops slowly as HEWL slowly loses its binding capacity (from 29% to 24% in the following 5 h), probably due to denaturing. The K_i , α value and β value are almost identical in both experiments (L-DOPA and L-tyrosine as substrates, respectively), which suggests this common inhibition mechanism affects both steps.

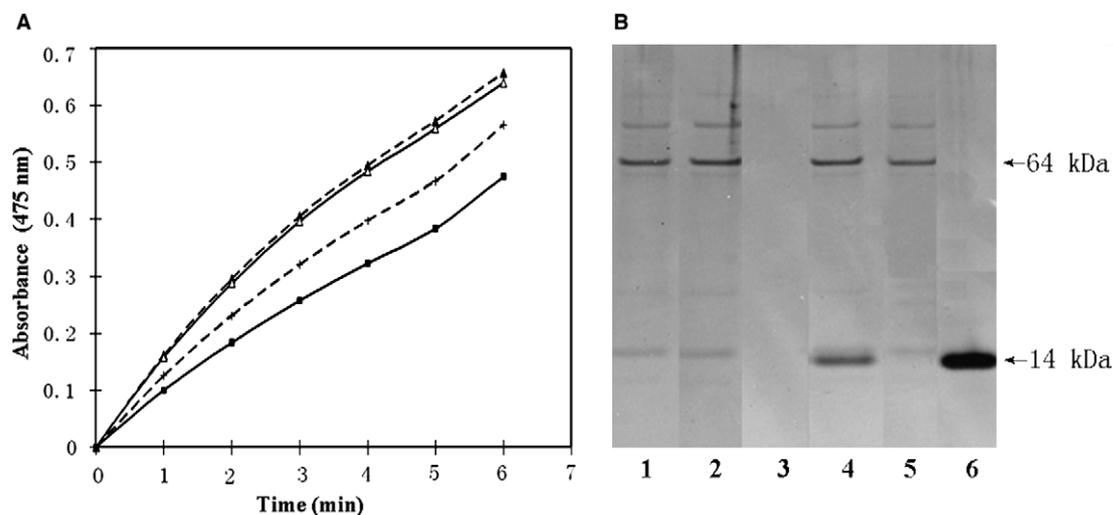


Fig. 4. HEWL depletion experiment: comparison of mushroom tyrosinase activity before and after HEWL depletion. (A) The recovery of inhibitory action of HEWL against mushroom tyrosinase after HEWL depletion by Sephadex CM beads. HEWL was added to mushroom tyrosinase and incubated for 1 h at 25 $^{\circ}\text{C}$. Mushroom tyrosinase alone (Δ , \blacktriangle) and tyrosinase/HEWL (\blacksquare , \square) were treated by Sephadex CM beads for 30 min (Δ , \square) or kept at 25 $^{\circ}\text{C}$ without HEWL depletion procedure (\blacktriangle , \blacksquare). The enzymes were dissolved in 50 mM MOPS (pH 6.5). (B) SDS-PAGE comparison of mushroom tyrosinase alone (lanes 1 and 2) and tyrosinase/HEWL (lanes 4 and 5) solution before (lanes 1 and 4) and after (lanes 2 and 5) Sephadex CM bead treatment. Proteins eluted from Sephadex beads after incubation with tyrosinase (lane 3) or with tyrosinase/HEWL mixture (lane 6). SDS-PAGE was performed using a 4–12% Bis-Tris gel with silver stain. Arrows indicate mushroom tyrosinase (64 kDa) and HEWL (14 kDa).

In summary, our study demonstrates for the first time HEWL acts as an inhibitor of mushroom tyrosinase by reversible inhibition and irreversible protein modification. Water-soluble and non-harmful HEWL as an inhibitor has the same (or even better) efficiency as some small molecule inhibitors. Taken together with our previous observations on the inhibition of mosquito melanin production by mosquito lysozyme and HEWL, these findings suggest that there is a universal mechanism of inhibition of tyrosinase by lysozyme, though its detail remains unclear. Future studies will be required to elucidate how lysozyme binds to and modifies tyrosinase at the structural level.

4. Materials and methods

4.1. Materials

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) was purchased from Acros Organics (Fair Lawn, NJ, USA). Mushroom tyrosinase, L-DOPA, Sephadex CM beads were purchased from Sigma (St. Louis, MO, USA). HEWL was purchased from Worthington Biochemical (Lakewood, NJ, USA). MOPS and L-tyrosine were obtained from Fisher Scientific (Houston, TX, USA).

4.2. Kinetic analysis of mushroom tyrosinase inhibition by HEWL

Mushroom tyrosinase activity was measured by determining its diphenolase activity using a modification of the method reported by Shono et al. [24]. HEWL was dissolved in MOPS buffer (50 mM, pH 6.5) at a range of concentrations from 0.167 to 1.33 μ M. Mushroom tyrosinase was prepared as 0.1 U/ μ l in MOPS buffer (50 mM, pH 6.5). The pre-incubation mixture consisted of 75 μ l of mushroom tyrosinase and 75 μ l of HEWL. The mixture was incubated at 25 °C for 3 h. The peak inhibition happened after 1-h pre-incubation. However, since the inhibition effect increased quickly before 1 h, the kinetics data around 1 h were sometimes not consistent. After 3 h, inhibition only decreased 10% from the maximum, but kinetics results were stable. Therefore, we chose the 3 h incubation period. Then 100 μ l of various concentrations of L-DOPA (3.33, 2.22, 1.48, 0.99, 0.5 and 0.25 mM) or L-tyrosine (4, 2, 1, 0.5 and 0.25 mM) in water was added to a 96-well plate (Becton Dickinson Labware, NJ, USA) for a total volume of 250 μ l for the assay mixture. The initial rate of dopachrome formation from the reaction mixture was determined as the increase of absorbance at 475 nm. Results are shown from three experiments. The pre-incubation of mushroom tyrosinase alone was treated as negative control. Kojic acid was used as a positive control. Sigma plot 2002 was used to process all kinetics data and to generate Lineweaver–Burk and Dixon plots for determination of K_i , ID_{50} , and the nature of the inhibition. The substrate concentrations we used to calculate the ID_{50} of HEWL inhibition of tyrosinase were 0.4 mM for L-DOPA and 0.8 mM for L-tyrosine. The substrate concentration we used for kojic acid inhibition of tyrosinase was 0.4 mM.

4.3. HEWL depletion experiment

Sephadex CM beads (carboxymethyl-Sephadex), a commercial polysaccharide studded with $-CH_2-COOH$ groups and used for chromatography were used for HEWL depletion. Above pH 3, these groups carry negative charges ($-CH_2-COO^-$), and thus will bind tightly to positively charged solutes [21]. The pI of HEWL is 11.0. At pH 6.5, the pH at which we carry out HEWL depletion experiment, the predominant ionic form of HEWL has a net positive charge, and thus HEWL will cling tightly to Sephadex CM beads. Our data show mushroom tyrosinase, which we wished to separate from HEWL, does not bind to the beads.

HEWL (75 μ l, 0.25 μ M) and mushroom tyrosinase (75 μ l, 7.5 U) were mixed for 1 h at 25 °C. After incubation, 8 μ l of a Sephadex CM bead slurry were added to the mixture and rotated for 30 min. The mixture was centrifuged; the supernatant and the protein attached on the beads (eluted by 1 M NaCl) were examined by SDS-PAGE (Fig. 4B). The diphenolase activity in the supernatant was compared with a mixture not subjected to Sephadex CM beads incubation

(Fig. 4A) by adding L-DOPA (100 μ l, 1 mM) as substrate after 1 h. Two controls of mushroom tyrosinase were designed to exclude the possibility that mushroom tyrosinase was accidentally inactivated or absorbed by Sephadex CM beads. The controls were incubated at 25 °C for 1 h, only one of which was followed by same HEWL-depletion procedure as above.

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