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Tyrosinase Kinetic Inhibition of Active Compounds from *Intsia palembanica*

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ABSTRACT

Intsia palembanica is the most potential plant having tyrosinase inhibitor over 30 Indonesian plants that have been searched. It's active compound namely (-)-robidanol, (+)-epirobidanol and 4'-dehydroxyrobidanol had been identified and showed the tyrosinase inhibitory activity. The study aims to obtain the kinetic enzyme inhibition type of the three active compounds. About 3 g of *I. palembanica* methanol extract was fractionated using n-hexane soluble part, ethyl-acetate soluble part and aqueous soluble part. Compounds were isolated for the most active part (ethyl-acetate soluble part) by silica gel column chromatography. Purification of fractions was performed using preparative High Performance Liquid Chromatography (HPLC). Kinetic inhibition against tyrosinase on monophenolase and diphenolase were analyzed by Lineweaver-Burk plot. The results showed that three active compounds showed different kinetic inhibition type on monophenolase and diphenolase. The kinetic characteristics of (-)-robidanol, the most active inhibitor, on monophenolase were K_m increased and V_{max} did not changed while on diphenolase were V_{max} decreased and K_m did not change. In conclusion, (-)-robidanol is a competitive inhibitor for monophenolase and a non-competitive inhibitor for diphenolase. The kinetic data is useful for further research on mechanism of action of the whitening agent that will be formulated.

Key words: *Intsia palembanica*, (-)-robidanol, tyrosinase, kinetic inhibition, monophenolase

INTRODUCTION

In the search for bioactive compounds with tyrosinase inhibitory activity from Indonesian medicinal plants, previously the research focused on *Intsia palembanica* (Batubara *et al*., 2010, 2011). Three active compounds isolated from *I. palembanica* had tyrosinase inhibitory activities, namely (-)-robidanol, (+)-epirobidanol and 4'-dehydroxyrobidanol (Fig. 1).

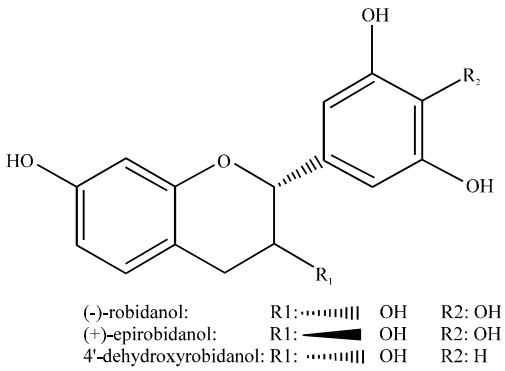

Fig. 1: Structure of three flavanols from *I. palembanica*

Since the active compounds reported have enzyme inhibitory activity, it is interesting to know the biochemical kinetic properties of an enzyme in the presence of active compounds. Biochemical kinetic properties like K_m and V_{max} are the biochemical properties most determined on an enzyme in the presence of some compounds or extracts, such as on adenosine deaminase in the presence of garlic and red clover (Avci *et al.*, 2005), catalase in the presence of *Brassica oleracea capitata* (Gholamhoseinian *et al.*, 2006), lysozyme in the presence of glyoxime and dichloroglyoxime (Ranjbar *et al.*, 2006), α-glucosidase in the presence of *Ficus deltoidea* (Adam *et al.*, 2010), alanine dehydrogenase in the presence of *Thielaviopsis paradoxa* (Al-Onazi *et al.*, 2011) and on $NADP^+$-linked-isocitrate dehydrogenase in the presence of *Phaseolus mungo* L. (Srivastava *et al.*, 2011). The kinetic parameters of tyrosinase such as K_m and V_{max} have also been reported such as the K_m and V_{max} values of tyrosinase isolated from potato and edible fungi (Yang and Wu, 2006).

Information about kinetics inhibition of tyrosinase inhibitor worth to determined, because there were many compounds natural and synthetic have activity of tyrosinase inhibitor but only some of them have the information about kinetics behavior (Chang, 2009). Enzyme inhibitors are classified into four types, namely competitive, uncompetitive, mixed (competitive/uncompetitive) and non-competitive inhibitors (Chang, 2009). Since the IC_{50} values, the usual expression for inhibitor strength, of the three flavanols isolated from *I. palembanica* were strong (Batubara *et al.*, 2011), it is interesting to find the kinetic inhibition of the compounds. The aim of this research was to classify inhibition strength of the three isolated flavanols from *I. palembanica*, by its kinetic inhibition on Lineweaver Burk plot.

MATERIALS AND METHODS

Plant materials, extraction, isolation and identification of active compounds: The research was performed from October 2010 till April 2011. *I. palembanica* samples were collected from Samarinda, East Kalimantan, Indonesia. All the extraction, isolation and identification processes were performed as in our previous report (Batubara *et al.*, 2010, 2011). Briefly, 3 g of *I. palembanica* methanol extract was fractionated using n hexane, ethylacetate and water to result to n-hexane soluble part, ethylacetate soluble part and aqueous soluble part. n-hexane soluble part (13 mg) had no tyrosinase inhibition activity, while ethylacetate soluble part (1.4 g) and aqueous soluble part (1.6 g) had tyrosinase activity (IC_{50} value for diphenolase were 3,97 and 4,34 ppm, respectively). Ethyl acetate soluble part which more active compared to aqueous soluble part was separated by silica gel column chromatography with hexane, ethyl acetate and methanol

as the developing solvents and resulted to 26 fractions. Fraction 4-8 purified using preparative HPLC with reversed phase column Inertsil ODS-3 (GL Sciences 10 mm id×200 mm) monitored at 280 nm. The solvent system used was as follows: a gradient program for 45 minutes from 5% to 100% of methanol in TFA 0.05% (in water) at flow rate of 3 mL min^{-1}. This separation step gave (-)-robidanol (15.8 mg), (+)-epirobidanol (47.4 mg) and 4'-dehydroxyrobidanol (10.5 mg) (Fig. 1). The structures of the isolated compounds were determined by comparison of their spectroscopic data with those reported in the literature. ^{1}H- and ^{13}C- NMR were recorded with a JEOL ECP 600 MHz spectrometer with TMS as the internal reference and chemical shifts expressed in δ (ppm). All spectroscopy data was reported on our previous report (Batubara *et al.*, 2011).

The kinetic inhibition test against mushroom tyrosinase: This assay was performed similar to the inhibition assay which we published previously (Batubara *et al.*, 2011). The concentration of pure compound used in this experiment was the same with IC_{50} (monophenolase: (-)-robidanol: 8.7 μM, (+)-epirobidanol: 20.2 μM, 4'-dehydroxyrobidanol: 15.2 μM; diphenolase: (-)-robidanol: 26.6 μM, (+)-epirobidanol: 178.5 μM, 4'-dehydroxyrobidanol: 50.0 μM). Concentrations of substrate were 0, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6 and 2.0 mM for L-tyrosine and 0, 1, 2, 4, 6, 8, 10 and 12 mM for L-DOPA. The Lineweaver and Burk plot of 1/Absorbance versus 1/[substrate] was used to determine the kinetic parameters of tyrosinase biocatalysis with or without the pure compound. The Michaelis constants Km and V max were calculated from the plot.

RESULTS AND DISCUSSION

(-)-Robidanol, (+)-epirobidanol and 4'-dehydroxyrobidanol are compounds in group of catechin like compounds, flavanol. Unlike catechin, the three isolated compounds are more active on monophenolase and diphenolase activities of tyrosinase (Batubara *et al.*, 2011). The kinetic type of catechin as tyrosinase inhibitor is a competitive type on monophenolase (Kim and Uyama, 2005) and mixed type inhibitor on diphenolase (Nirmal and Benjakul, 2012).

The inhibition kinetics of (-)-robidanol, (+)-epirobidanol and 4'-dehydroxyrobidanol were analyzed using Lineweaver-Burk plot on their IC_{50} values. The results for monophenolase and diphenolase activities are presented in Fig. 2 a and b, respectively. In monophenolase activity (substrate: L-tyrosine), K_m value of (+)-epirobidanol was increased while V_{max} decreased. The results showed that (+)-epirobidanol is a mixed inhibitor type. Unlike (+)-epirobidanol, (-)-robidanol and 4'-dehydroxyrobidanol are competitive inhibitors because the K_m increased while V_{max} did not changed. The inhibition type of (-)-robidanol and 4'-dehydroxyrobidanol are the same with kojic acid on monophenolase activity (Chen *et al.*, 1991), catechin (Kim and Uyama, 2005) and gallocatechin-3-O-gallate (No *et al.*, 1999). Different with the isolated compounds and kojic acid, sesamol from sesame seed had been reported as a non-competitive inhibitor on monophenolase (Kumar *et al.*, 2011).

In diphenolase (substrate L-DOPA), the inhibition type of (+)-epirobidanol and 4'-dehydroxyrobidanol are uncompetitive type because K_m value and V_{max} decrease, while (-)-robidanol is non-competitive inhibitor (V_{max} decreased and K_m did not change). The types of inhibition of 3 isolated compounds on diphenolase are different with catechin, a mixed type inhibitor which could bind with enzyme and enzyme-substrate complex of tyrosinase (Nirmal and Benjakul, 2012). Most information relating to the type of inhibitor for diphenolase are existing

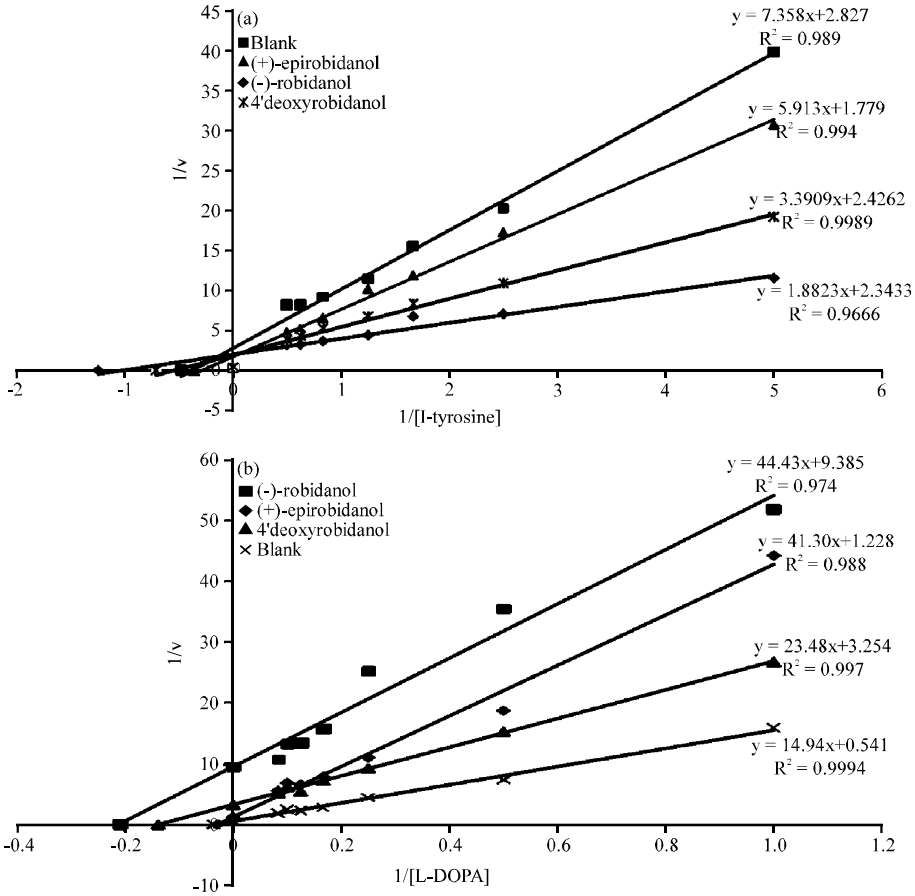

Fig. 2(a-b): Lineweaver-Burk plot of active compounds from *I. palembanica* on (a) monophenolase and (b) diphenolase

especially as a competitive inhibitor, for example, flavonols such as quercetin, galangin, fisetin, 3,7,4'-trihydroxyflavone and morin (Xie *et al*., 2003), flavones such as norartocarpetin (Ryu *et al*., 2008), isoflavones such as 6-hydroxydaidzein (Chang *et al*., 2007) and also sesamol, a phenolic compound formed from mushroom (Kumar *et al*., 2011). The data of inhibition type of 3 isolated compounds from *I. palembanica* added the type of inhibitor for diphenolase activity of tyrosinase.

The inhibition kinetics of monophenolase and diphenolase are different because tyrosinase consists of two active sites. The formations on monophenolase and diphenolase reactions were different. On monophenolase reaction, hydrophobic interaction reaction in substrate took place between one Cu atom on tyrosinase while on diphenolase reaction, hydroxyl group of L-DOPA will be deprotonated by peroxide ion bound between the two Cu atoms in tyrosinase (Ismaya *et al*., 2011).

Competitive inhibitor of tyrosinase means that the inhibitor binds with free enzyme. On the other hand, competitive inhibitors also can prevent substrate to bind with enzyme or inhibitor can be a copper chelator. The enzyme-substrate complex binding only showed for an uncompetitive inhibitor type. While for a mixed type inhibitor can bind to a free enzyme and also to the enzyme-substrate complex with a free enzyme (Chang, 2009).

CONCLUSION

The three active compounds showed different kinetic inhibition type on monophenolase and diphenolase. The most active compound, (-)-robidanol, had a competitive inhibitor type for monophenolase and a non-competitive inhibitor type for diphenolase.

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