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Vol. 14(29), pp. 2321-2326, 22 July, 2015
DOI: 10.5897/AJB2015.14651
Article Number: 9D56A3A54262
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Biochemical and kinetic characterization of geranylgeraniol 18-hydroxylase (CYP97C27) from *Croton stellatopilosus* Ohba

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Received 18 April, 2015; Accepted 13 July, 2015

Geranylgeraniol 18-hydroxylase (EC 1.14.13.110) that exists solely in *Croton stellatopilosus* Ohba catalyses the last committed step of plaunotol biosynthetic pathways by conversion of geranylgeraniol (GGOH) to plaunotol. This enzyme and its gene are an attractive target for development of plaunotol production and its detailed biochemical properties need to be understood. Recently, even though the gene (*CYP97C27*) coding for GGOH 18-hydroxylase has been identified, cloned, and expressed in *Escherichia coli* system, the enzyme activity has been detected mainly in the insoluble fraction (20,000 *g*). This means that biochemical and kinetic studies could not be undertaken. However, our previous study indicated that this enzyme activity was easily and specifically detected in the microsomal fraction (100,000 *g*) of a crude enzyme extract. Therefore, in this report we describe a comprehensive biochemical characterization of GGOH 18-hydroxylase activity in the microsomal fraction from *C. stellatopilosus* Ohba. The oxygen-dependent enzyme activity of GGOH 18-hydroxylase was inhibited by carbon monoxide and the inhibition was partially reversible upon illumination with white light. Kinetic studies of the GGOH 18-hydroxylase showed high affinity to GGOH and NADPH with apparent K_m values of 0.8 and 53 µM, respectively. Furthermore, the enzyme activity was inhibited by P450 inhibitors, including ancymidol, metyrapone, miconazole, potassium cyanide and cytochrome c, with the IC_{50} values of 428, 65, 75, 66 and 8 µM, respectively. Based on the biochemical and kinetic characteristics, the GGOH 18-hydroxylase in the microsomal fraction is likely a P450 encoded by *CYP97C27* gene as previously described.

Key words: Plaunotol, cytochrome P450, enzyme activity, enzyme inhibitor, microsome.

INTRODUCTION

Plaunotol, an acyclic diterpenoid compound, has important chemotherapeutic activities; it exhibits an anti-inflammatory action in association with peptic ulcers and antimicrobial activities against *Helicobacter pylori* and *Staphylococcus aureus* (Koga et al., 2002; Inoue et al., 2004; Premprasert et al., 2013). It was originally found in

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Abbreviations: GGPP, Geranylgeranyl diphosphate; **GGOH,** geranylgeraniol; **PMSF,** phenylmethylsulfonyl fluoride; **KCN,** potasium cyanide; **CO,** carbon monoxide.

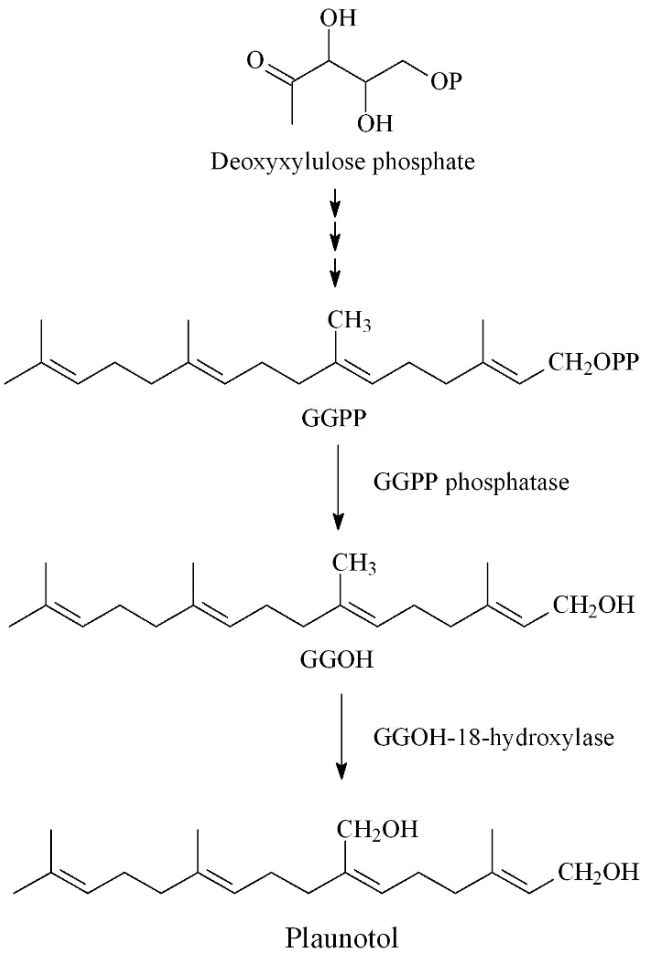

Figure 1. The biosynthetic pathway of plaunotol in *C. stellatopilosus* via deoxyxylulose phosphate pathway (modified from Nualkaew et al., 2006).

leaves and stems of *Croton stellatopilosus* Ohba (Euphorbiaceae), a medicinal plant growing in tropical Southeast Asian countries, especially Thailand. It appears to be accumulated mainly in the chloroplasts (Wungsintaweekul and De-Eknamkul, 2005; Sitthithaworn et al., 2006). Previous study on plaunotol biosynthesis in *C. stellatopilosus* has revealed that biosynthesis of plaunotol was carried out via the deoxyxylulose phosphate pathway in which geranylgeranyl diphosphate (GGPP) and geranylgeraniol (GGOH) were the intermediate precursors (Nualkaew et al., 2005; Wungsintaweekul and De-Eknamkul, 2005). As shown in Figure 1, GGPP is dephosphorylated by phosphatase to form GGOH, then the GGOH is subsequently hydroxylated at the C-18 position by GGOH 18-hydroxylase (EC 1.14.13.110) to form plaunotol, where the hydroxylation of GGOH exhibits the requirement of NADPH as a reducing equivalent of the reaction (Tansakul and De-Eknamkul, 1998; Nualkaew et al., 2005; Nualkaew et al., 2006). This suggested that the GGOH 18-hydroxylase is a member of cytochrome P450 monooxygenases (CYP), similar to other P450 hydroxylases involved in plant secondary metabolisms (Collu et al., 2001; Jennewein et al., 2003). As yet, there are no more details with regard to the biochemical and kinetic properties of GGOH 18-hydroxylase although the corresponding gene (*CYP97C27*) has been identified, cloned and expressed in an *Escherichia coli* system (Sintupachee et al., 2014). The expressed GGOH 18-hydroxylase protein was highly detectable in the insoluble fraction of the crude enzyme, however it was difficult to obtain in highly purified solubilized form and thus it was not amenable to detailed characterization. However, all P450 enzymes in plants have been reported to be localized to the microsomal fraction (Schuler, 1996) and the activity of our enzyme of interest (GGOH 18-hydroxylase) has been shown to exist in the 100,000 *g* microsomal pellet fraction of crude enzyme extract. Therefore, in this work, we investigated the biochemical and kinetic properties of GGOH 18-hydroxylase in the 100,000 *g* microsomal fraction.

MATERIALS AND METHODS

Plant materials and chemicals

Fresh mature leaves of *C. stellatopilosus* Ohba were kindly provided by the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand, and maintained at -20°C until used. All chemicals were of the highest purity available. Geranylgeraniol (GGOH), β-nicotinamide adenine dinucleotide phosphase (reduced form) tetrasodium salt (NADPH), β-mercaptoethanol, ancymidol, metyrapone, miconazole and cytochrome c were purchased from Sigma Chemical Co., (USA). Tricine and dithiothreitol (DTT) from USB (USA). Phenylmethylsulfonyl fluoride (PMSF) and potasium cyanide (KCN) from Fluka (Switzerland). Ethyl acetate and absolute ethanol were of HPLC grade and purchased from Lab-Scan Asia (Thailand). Plaunotol was obtained from Kelnac® soft gelatin capsules (Sankyo, Co., Japan).

Preparation of microsomal fraction containing GGOH 18-hydroxylase activity

All enzyme preparation steps were performed at 0 to 4°C. The GGOH 18-hydroxylase was prepared and purified according to the method as previously described (Chanama et al., 2009). Briefly, 30 g of frozen mature leaves were ground rapidly to a powder in liquid nitrogen using a prechilled mortar and pestle. Then, the fine powder was extracted in 60 ml of extraction buffer containing 83 mM Tricine-NaOH (pH 7.8), 0.4 M sucrose, 10 mM EDTA, 10 mM $MgCl_2$, 10 mg/ml BSA, 1 mM DTT, 0.8 mM PMSF and 5 mM β-mercaptoethanol, and the mixture was stirred for 10 min. The homogenate was filtered through several layers of cheesecloth, centrifuged at 3,000 *g* for 10 min and the supernatant collected prior to spinning for 20 min at 20,000 *g*. Resulting supernatant was further ultracentrifuged at 100,000 *g* for 60 min and the microsomal precipitate was suspended in 3 to 5 ml of 0.1 mM Tricine-NaOH (pH 7.8) containing 0.2 M sucrose, 1 mM EDTA, 1 mM DTT, 15% glycerol and 5 mM β-mercaptoethanol. The final enzyme preparation containing the GGOH 18-hydroxylase was stored at -80°C, and protein concentration of the enzyme was measured using the Bio-Rad protein assay (Bradford, 1976).

GGOH 18-hydroxylase activity assays

Enzyme activity of GGOH 18-hydroxylase was assayed as previously described (Chanama et al., 2009). The reaction mixture consisted of 83 mM Tricine-NaOH (pH 7.8), 0.8 mM NADPH and 57 µM GGOH (substrate). The reaction was initiated by the addition of 100 µl of enzyme preparation (~ 250 µg of protein) to the mixture. The reaction mixture was incubated at 30°C for 30 min and then stopped by extraction twice with an equal volume of ethyl acetate. The extracts were pooled and dried, and the residue was redissolved in ethyl acetate for analysis of plaunotol. Plaunotol content in the extract was determined using a TLC-densitometric technique as previously described. Briefly, the extract of enzyme product was applied onto silica gel_{60} F_{254} plates (CAMAG) using Linomat IV (CAMAG). The plate was developed by ethyl acetate and scanned at wavelength of 210 nm to obtain chromatogram of the sample. The plaunotol content was then estimated on the basis of the standard calibration curve of pure plaunotol compound. The assays were performed in triplicate. The enzyme activity is expressed as katal unit (kat). One katal is the amount of enzyme required to convert GGOH substrate to one mole of plaunotol product per second. For kinetic studies of the GGOH 18-hydroxylase, the maximum velocity (V_{max}) and the Michaelis constant (K_m) values were determined under the standard assay conditions with substrate concentrations ranging from 0.2 to 10 µM for GGOH and from 0.02 to 1.0 mM for NADPH.

Inhibition experiments

The inhibition by gaseous carbon monoxide (CO) and reversal of CO inhibition by white light were performed in 2 ml septum-capped glass vials containing all the reaction components except GGOH and NADPH. The vials were placed on ice, and each gas (CO, N_2 or air) was bubbled through the reaction mixtures. The reactions were initiated by the addition of GGOH and NADPH, and then incubated at 30°C for 30 min in the dark or under white light (for light reversal of CO inhibition). After incubation, the reaction products were extracted and analysed as described above. The control vials without the addition of inhibitors were carried out in the dark. The cytochrome P450 inhibitors: ancymidol, metyrapone, miconazole, KCN and cytochrome c were tested. The concentrations of ancymidol, metyrapone, miconazole and KCN were adjusted from 0.01 to 6.0 mM and cytochrome c from 0.003 to 0.3 mM. The enzyme inhibition tests were carried out by addition of the inhibitors to the standard assay. After completion of the reactions, the enzyme products (plaunotol) were extracted and analysed by TLC. The inhibition is expressed as IC_{50} value, that is, the concentration of an inhibitor where the enzyme activity is reduced by 50%.

RESULTS AND DISCUSSION

The enzyme GGOH 18-hydroxylase found in *C. stellatopilosus* catalyses the conversion of GGOH to plaunotol by addition of hydroxyl group (-OH) to acyclic diterpenoid GGOH at position of C-18 and the hydroxylation reaction is highly specific to this acyclic diterpene substrate and not for other terpenoid compounds (C-10: geraniol, C-15: farnesol) (Tansakul and De-Eknamkul, 1998). In addition, the activity was determined 1.5-fold higher in 100,000 *g* microsomal fraction than in the 20,000 *g* insoluble fraction under the presence of NADPH and aeration (Chanama et al., 2009) and also observed in other plant sources (Bolwell et al., 1994, Pierrel et al., 1994). In this work, we studied the kinetic properties of the GGOH 18-hydroxylase enzyme in the 100,000 *g* microsomal fraction with its substrate and cofactor. The results showed that the GGOH 18-hydroxylase was saturated at approximately 7 µM GGOH and 500 µM NADPH, and apparent K_m values for the hydroxylation of GGOH and for NADPH were 0.8 and 53 µM, respectively (Figure 2). The very low K_m value of the hydroxylase for GGOH implies the high affinity of the GGOH 18-hydroxylase for the GGOH substrate, and this result is in good agreement with the K_m values obtained for the hydroxylation of laurate by lauric acid (P450) monooxygenase from Jerusalem-Artichoke (*Helianthus tuberosus*) (0.97 µM) (Salaun et al., 1978) and from wheat (*Triticum aestivum*) (8 µM) (Zimmerlin et al., 1992). Interestingly, the apparent K_m value of this enzyme for diterpenoid GGOH was 20 to 60 fold lower than those for the hydroxylations of other fatty acid (16-hydroxypalmitic acid, 50 µM), acyclic monoterpene (geraniol, 15 µM), cyclic monoterpene (limonene, 18 to 21 µM), cyclic diterpene (taxoid, 50 µM) and phenolic compound (cinnamic acid, 35 µM) from other plant species (Soliday and Kolattukudy, 1978; Karp et al., 1990; Hallahan et al., 1992; Petersen, 1997; Jennewein et al., 2003). In addition, consumption of NADPH in the hydroxylation is essential for the enzyme activity and in a good agreement with the result reported by Tansakul and De-Eknamkul (1998). The K_m value observed in this study certainly supports the view that GGOH and NADPH are actual substrate and cofactor in the enzyme system.

To investigate whether the GGOH 18-hydroxylase present in the microsomal fraction belongs to a member of cytochrome P450 monooxygenase, several of the following criteria, that is, a requirement for molecular oxygen (O_2), inhibition by CO and reversal of the CO inhibition by light, and inhibition by specific cytochrome P450 inhibitors were examined (Krochko et al., 1998; Kim et al., 2004). To address whether molecular oxygen (O_2) was essential for GGOH 18-hydroxylase activity, replacement of dissolved oxygen in the reaction mixture by nitrogen gas prior to initiating reaction was performed. The reaction exhibited a dramatic decrease in enzyme activity (85% inhibition) (Table 1). Moreover, carbon monoxide, which is known to bind effectively to Fe(II)-heme region of the cytochrome P450 (Krochko et al., 1998), also exhibited the strongest inhibition when it was introduced into the enzyme system in the dark. The relative activity of GGOH 18-hydroxylase inhibited by CO was reduced to as low as 5.87% of control (94% inhibition). This inhibition was partially reversible upon illumination with visible light (relative activity of 35%) as shown in Table 1. This partial reversion of CO-inhibition by white light (particularly at wavelength of 450 nm) could be due to the photolysis of Fe (II)-CO complex which exists in the GGOH 18-hydroxylase. The effects of specific cytochrome P450 inhibitors (ancymidol,

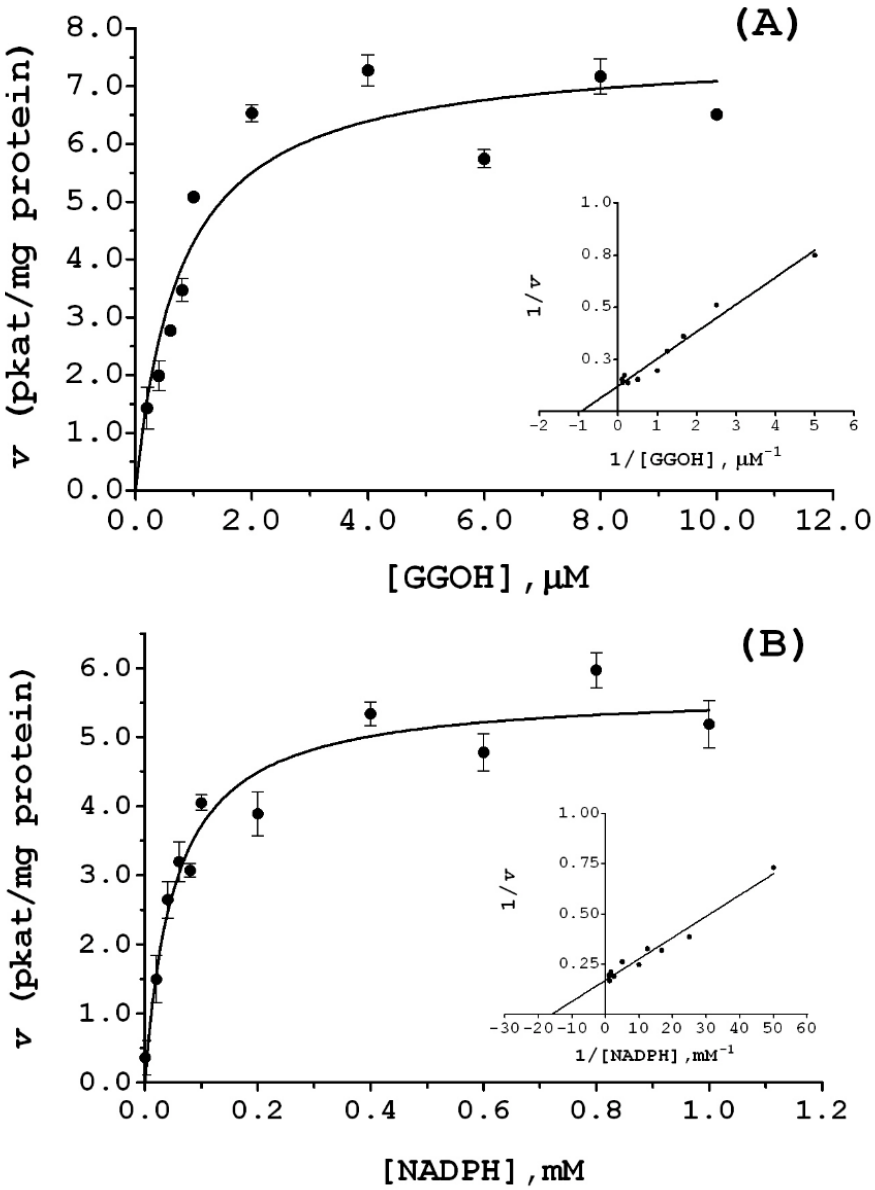

Figure 2. Michaelis-Menten plots showing the variation of initial velocity of GGOH 18-hydroxylase reaction as a function of GGOH (A) and NADPH (B) concentrations. The double reciprocal plots 1/V as a function of 1/[GGOH] and of 1/[NADPH] are shown in the insets. The values are means (±SEM) of three separate experiments.

Table 1. Effect of oxygen (air), nitrogen (N_2) and carbon monoxide (CO), and white light on the activity of GGOH 18-hydroxylase from *C. stellatopilosus*.

Treatment	Relative activity [a] (% of control)	Inhibition (%)
Control (air, dark)	100.0	00.00
N_2	14.47	85.53
CO (dark)	05.87	94.13
CO (white light)	35.56	64.44

[a]The enzyme activity of the control was 1.77 pkat/ mg protein.

metyrapone, miconazole, cytochrome c and KCN) were used in this study. All cytochrome P450 inhibitors inhibited the enzyme activity of GGOH 18-hydroxylase completely or to a lesser extent and with variable concentrations for half-maximal activity (IC_{50} values) (Figure 3). Ancymidol, metyrapone, miconazole and KCN exhibited 100% inhibitory effects on the hydroxylation reaction at concentrations ranging from 0.01 to 6 mM with IC_{50} values of 428, 65, 75 and 66 µM, respectively.

On the other hand, cytochrome c which is known to remove electrons competitively from NADPH-cytochrome

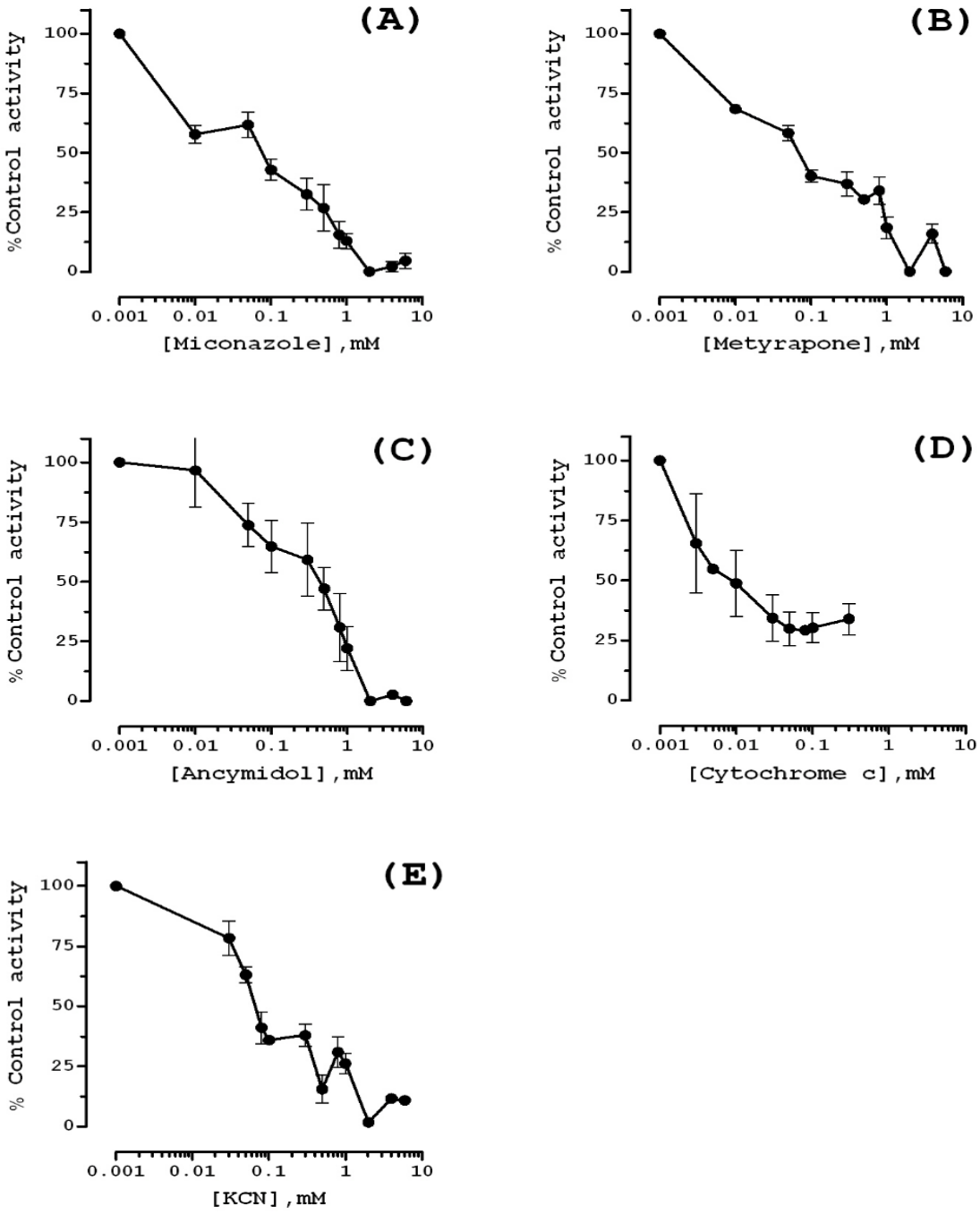

Figure 3. Inhibitions of GGOH 18-hydroxylase activity by cytochrome P450 inhibitors. The values are means (±SEM) of three separate experiments.

P450 reductase complex (Petersen, 1997), showed about 75% inhibition with the lowest IC_{50} of 8 µM. Among these inhibitors tested, cytochrome c seemed to be the most potent inhibitor against GGOH 18-hydroxylase (IC_{50} of 8 µM). These results strongly support a typical feature of plant cytochrome P450s of the enzyme (Friederich et al., 1999; Yamamoto et al., 2000; Katano et al., 2001). According to the biochemical and kinetic properties of the GGOH 18-hydroxylase, that is, high affinity to GGOH substrate, requirement for molecular oxygen and NADPH, inhibition by P450 inhibitors, inhibition by CO, and reversal of the CO inhibition by light, it is likely that the GGOH 18-hydroxylase in the 100,000 *g* microsomal fraction of *C. stellatopilosus* is the CYP97C27 previously identified by RT-PCR methodology from leaves of *C. stellatopilosus* (Sintupachee et al., 2014).

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by Chulalongkorn University and Mahidol University. The authors would like to thank the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University for leaves of *C. stellatopilosus* Ohba. This study was supported for publication by the China Medical Board (CMB), Faculty of Public Health, Mahidol University, Bangkok, Thailand.

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