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Determination of the Kinetic Properties of Platycodin D for the Inhibition of Pancreatic Lipase Using a 1,2-Diglyceride-Based Colorimetric Assay

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A 1, 2-diglyceride-based multi-step colorimetric assay to measure the pancreatic lipase activity was applied for the determination of the kinetic profiles of the lipase inhibition with a slight modification and the validity verification. With this assay method, our study revealed that platycodin D, one of major constituents of Platycodi Radix, inhibits the pancreatic lipase activity in a competitive type, with the value of K_i being 0.18±0.02 mM. In addition, PD has affected the values of $K_{m, app}$ and K_{cat}/K_m in a dose- dependent manner. The results shed a meaningful light on how PD mediates lipid metabolism in the intestinal tracts. On the other hand, since the revised assay is sensitive, rapid, and does not affect the accuracy to the kinetic properties, it is applicable not only to evaluation of the kinetic properties of the pancreatic lipase, but also to high-throughput screening of pancreatic lipase activity.

Keywords: platycodin D, pancreatic lipase, 1, 2-diglyceride, K_i, K_{cat}/K_m

INTRODUCTION

Pancreatic lipase (E.C 3.1.1.3) is an enzyme that is produced in the pancreas (Farrigan and Pang, 2002; Ros, 2000). Pancreatic lipase plays a key role in lipid digestion, catalyzing triglycerides to progressively release diglycerides, monoglycerides, and free fatty acids. Pancreatic lipase is also a favored marker of the acute pancreatitis in clinical diagnosis. Because of its importance in lipid digestion, the inhibition of pancreatic lipase is regarded as a practical approach in investigating the anti-hyperlipidemia and anti-obesity (Drent and Van der Veen, 1993; Farrigan and Pang, 2002; Keating and Jarvis, 2001; Wilding, 2000). To date, of a wide variety of assays that are used to detect lipase activity, two concepts have been principally applied. The first concept is to measure the released free fatty acids either by titrating with NaOH (Chung J, 1974; Tietz *et al.*, 1989) or by extracting with organic solvents and subjecting them to chromogenic reactions (Han *et al.*, 2001). But this type of method has a common drawback of being too sophisticated to conduct as an automatic operation, and accordingly, it would be difficult to achieve with high precision, unless a special continuous pH-stat titration apparatus was applied (Chung, 1974). The second concept is to measure the intrinsic chromophores that are released from the cleavage of the synthetic substrates (Burdette and Quinn, 1986; Panteghini *et al.*, 2001; Uusitupa, 1999). But since most of these type of assays have used non-pancreatic lipases as enzyme sources and the synthesized *p*-nitrophenyl esters as the substrate (Burdette and Quinn, 1986; Pencreac'h *et al.*, 2002), the results don't always reflect the situation of the pancreatic lipase.

On the other hand, for the purpose of clinical diagnosis, the multi-enzymatic method (Imamura, 1989) has become overwhelmingly popular in the last decade. In this method, 1, 2-diglyceride is used as the substrate and is catalyzed by a series of enzymes in turn and finally a colorimetrically detectable end-product, quinine dye, is generated (Fig. 1). This assay is sensitive, rapid and easily adaptable to automated measurement, but the use of other enzymes followed by the lipase, generally limits its application in the estimation of the lipase inhibition kinetics. According to our present study, however, the above method in case that PD was used as an inhibitor, was proved to be valid for determining the kinetic profiles of the pancreatic lipase.

Platycodin saponins (PS) are the primary constituents

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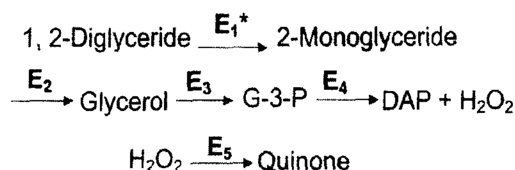

Fig. 1. The cascade reactions for lipase activity assay. E1* represents pancreatic lipase, a unique rate-limiting step, which catalyzes 1, 2-diglycerides to release monoglyceride. E2-5 represent in sequence, monoglyceride lipase, glycerol kinase, glycerol-3-phosphate oxidase, and peroxidase. They are responsible for a prompt transfer of the released glycerol to form a chromophoric end-product. The activities of E2-5 are higher than that of E1 by 100-4000 fold under the assay conditions. DAP (dihydroxyacetone phosphate), G-3-P (glycerol-3-phosphate).

and bioactive entities of Platycodi Radix, whose pharmacological actions have been well acknowledged by traditional Chinese medicines as an expectorant for pulmonary disease and a remedy for respiratory disorder. Recently, it has gained a renewed interest for its potential application in the control of anti-obesity and hypolipidemia (Han *et al.*, 2000; Hiroahi, 1981). Platycodin D (PD), one of the major components of PS, was once reported to possess an inhibitory effect toward the pancreatic lipase *in vitro* (Han *et al.*, 2002), but no detailed kinetic profiles were reported. In the present study, we managed to determine the kinetic parameters with a small quantity of PD using our modified assay. The results indicate that PD inhibits pancreatic lipase and it is likely to be one of the factors responsible for the anti-obese and hypolipidemic effects.

MATERIALS AND METHODS

Purification of PD

The raw sample (10 kg) of Platycodi Radix was extracted with methanol and was partitioned sequentially with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. Then, the butanol fraction was subject to Diaion HP-20 resin (Mitsubishi Chemical Corporation, Japan), and the fractions eluted at 50-100% of methanol was collected to obtain 45 g of crude saponins. The crude saponins were further purified by repeated silica gel (Merck, Germany) chromatography to obtain 220 mg of purified PD. PD was identified on the basis of the R_f, FAB-MS (=1225.38) and ^{13}C-NMR spectrum.

Measurement of pancreatic lipase activity

Lipase-PS™ Diagnostic kits were purchased from Sigma Aldrich (St. Louis, MO), which includes LIPASE (containing 250 units/l of pancreatic lipase), SUB (containing 1.1 mM of 1, 2-diglyceride, co-lipase and other pertinent enzymes), and ACTIVATOR (containing 36 mM of deoxycholate).

The measurement of lipase activity and IC_{50} of PD was processed as follows. In brief, the reagents of LIPASE (30 mg/ml) and SUB (1 mM) were fully mixed in advance at the ratio of 1:20 (v/v), and onto an aliquot of 95 µl of the above mixed solution, five microliters of 0, 0.2, 0.4, 0.8, 1.6 mM of PD was added, mildly mixed, and incubated in 25°C for 3 min. Then, the reaction was initiated by adding 25 µl of ACTIVATOR. The linear increase of the absorbance at 550 nm was read with an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA) at one minute interval each.

Measurement of the kinetic constants

In order to measure the apparent Michaelis constant, K_m, and the inhibition constant, K_I, a series of SUB concentrations were added into the assay system. Those unmentioned conditions were identical as described above. Each analysis was performed with or without inhibitors (PD = 0, 0.8, 1.6 mM). To measure the value of turnover, K_{cat}, the reaction rates at the condition of $[S] << K_m$ were measured with or without inhibitors (PD = 0, 0.8, 1.6 mM). The unmentioned assay conditions were identical as described above.

Validity of the specificity for the lipase inhibition

A lipase "knockout" assay was designed to verify if PD specifically inhibited only lipase activity, where 50 mM of glycerol was directly added instead of lipase, and the other reagents were left unaltered. The assays (glycerol$^+$ lipase$^-$) were performed as the time course change of the absorbance (550 nm) with or without PD (PD = 0, 0.8, 1.6 mM). The normal activity assays (glycerol$^-$ lipase$^+$) with or without PD were also shown for comparison.

One unit of lipase activity was defined as the liberation of 1 µmol of free fatty acid per minute under the assay conditions.

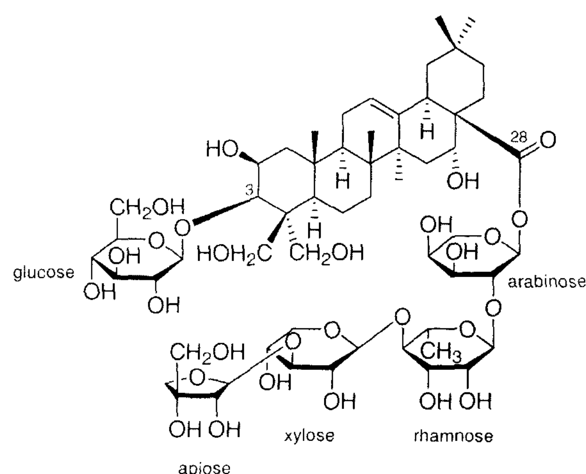

Fig. 2. Structure of platycodin D. Platycodin D is a triterpenoid bidesmoside, which is composed of aglycone moiety and two glycosyl side chains.

Data analysis

Using the Sigma plot 2000 software (IL, USA), the values of the reaction rate were obtained from the slope of each time course curve against the reaction process. Lineweaver-Burk plots were fitted to determine the apparent Michaelis-Menten constants for pancreatic lipase using 1, 2-glyceride as the substrate. The inhibition constant, K_I was calculated from the following equation:

$$K_{m,\,app} = K_{m,\,app'}\,(1 + [I] / K_I)$$

Where $K_{m,\,app'}$ and $K_{m,\,app}$ represent the apparent Km with or without PD. [I] represents the concentration of PD.

The value of K_{cat} was directly calculated from the reaction rates obtained when [S] << K_m, whereby the reaction is of the first order.

RESULTS AND DISCUSSION

The kinetic properties of PD for lipase inhibition

The kinetic properties for PD inhibition of the lipase activity were listed in Table 1. Under our assay conditions, IC_{50} of PD was determined to be 2.1 mM (Fig. 3). The value of the apparent K_m without PD was 0.38 mM, and with the addition of 0.8 and 1.6 mM of PD, the values shifted to 2.17 and 4.50 mM, respectively. The K_I value of PD was 0.18 ± 0.03 mM (based on the results from Fig. 4). As the concentration of PD varied (Fig. 4), the value of y-intercept ($1/V_m$) of each curve remained at a fixed point, which indicated that PD inhibition to pancreatic lipase was a competitive type. The value of V_m was 10.4 μM /min.

K_{cat} is used to describe the limiting rate of any enzyme-catalyzed reaction at saturation. If there are several steps in the reaction and one is clearly rate-limiting, K_{cat} is

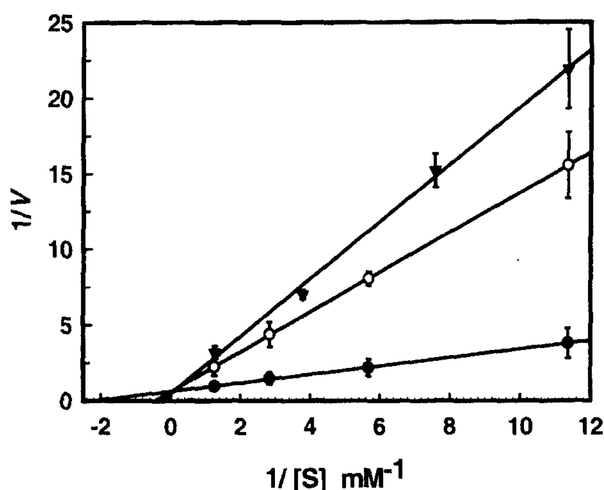

Fig. 3. Lineweaver-Burk plots for the lipase inhibition. The analysis was performed in triplicate ($r > 0.99$) with the concentration of PD as 0 mM (●), 0.8 mM (○), and 1.6 mM (▼), respectively. The reaction rate was relatively expressed being compared to that of V_m.

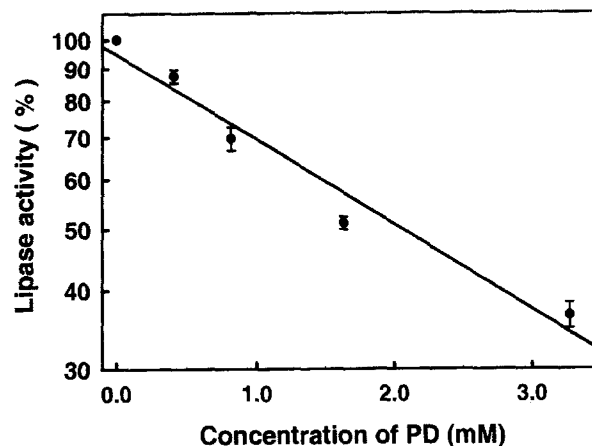

Fig. 4. The lipase inhibition of platycodin D. One hundred microliters of the mixed solution of LIPASE, SUB, and PD of given concentrations were loaded in the multi-well microplate and were incubated at 25°C for 3 min. The reaction was initiated by adding 25 μL of ACTIVATOR. The reaction rate was measured by continuously detecting the increase of the absorbance at 550 nm, and the rate was relatively expressed as compared to the value of V_m. The analysis for IC_{50} value was performed in triplicate. The remaining lipase activity (%, Y-axis) was expressed as a logarithm scale type.

equivalent to the rate constant for that limiting step (David *et al.*, 2000). In this assay system, lipase is the unique rate-limiting step (Fig. 1), and therefore, the K_{cat} values measured here represent the catalytic profiles of the lipase only and are irrelevant to the followed reactions. As shown in Table I, the values of K_{cat} decreased drastically from 7.01 ± 0.66 min^{-1} in the absence of PD, to 3.01 ± 0.43 min^{-1} and 2.97 ± 0.34 min^{-1}, respectively, in the presence of 0.8 and 1.6 mM of PD. This illustrates that PD's inhibition for the rate-limiting step was effective. As the concentra-tion of PD varied, an almost identical magnitude of lipase inhibition was obtained. It could be explained by the fact that the magnitude of the competitive inhibition is closely interrelated with the ratio of [S]/[I]. When [I] was nearly saturated with the given [E], the inhibition magnitude was not sensitively influenced by [I]. When the ratio of [S]/[I] steadily increases, the inhibition magnitude would turn more sensitive to [I] and thus become more perceptible, as the typical Michaelis-Menten curve characterizes.

The influence of the inhibition could be depicted in a more visible mode by using the ratio of K_{cat} / K_m (Table I). The values of K_{cat} / K_m declined in a dose dependant manner from 17.6 $\mu M^{-1} min^{-1}$ to 1.54 and 0.78 $\mu M^{-1} min^{-1}$ in the presence of 0.8 or 1.6 mM of PD, respectively.

The specificity of the assay for the lipase inhibition

The assay system contained 1, 2-diglyceride as the substrate for the lipase (rate-limiting step) to release the

Table I. Kinetic properties of PD for pancreatic lipase inhibition

PD [mM]	K_m [mM]	K_{cat}† [min^{-1}]	K_{cat}/K_m [μM^{-1}·min^{-1}]
0	0.38	7.01± 0.66	17.9
0.8	2.17	3.01 ± 0.43	1.54
1.6	4.50	2.97± 0.34	0.78

†The values of K_{cat} were directly calculated from the resulting linear time-course curves of the reaction rates at [S] << K_m and they represent presence and absence of PD. The experiments were performed in duplicate. The linear correlation *r* for each curve was > 0.99.

glycerol, in which the release was followed by the rapid depletion of glycerol by a series of enzymes to generate the detectable end-product (Fig. 1). Since the end products were generated from a series of enzymatic reactions, the assay of lipase inhibition is valid only when PD specifically acts on the lipase and not on the following steps. Therefore, a special assay was conducted, in which 0.05 M of glycerol (this concentration is 50 fold higher than that of the glycerol the lipase can generate) was added instead of the lipase (G^+/L^-, *i.e.* in the presence of glycerol but the absence of lipase, Fig. 5). The reaction proceeded promptly (within several seconds) and was not affected by the addition of PD (0, 0.8, 1.6 mM) at all. In contrast, in the presence of lipase and absence of glycerol (G^-/L^+), the reaction rate turned much slower, sustained at least 20 min, and displayed a dose-dependent inhibition to different PD concentrations.

Collectively, PD acted on the reactions of the glycerol generation that was catalyzed by the lipase and did not on that of glycerol elimination catalyzed by the other enzymes. This validity verification is essential because this assay

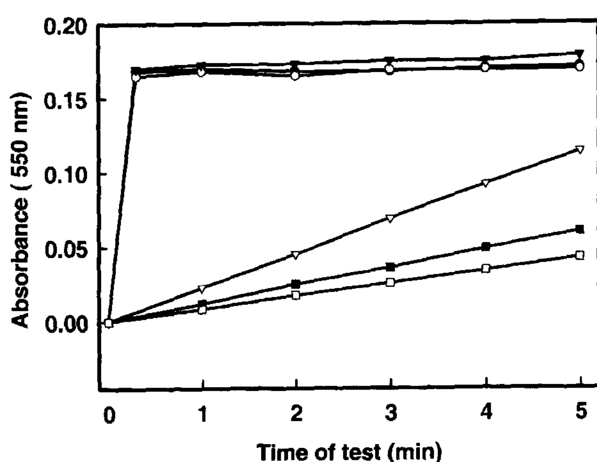

Fig. 5. Comparison of the reactions with or without lipase. The reactions were sequentially performed in the presence of glycerol and absence of lipase (G^+/L^-) with the concentration of PD as 0 mM (●), 0.8 mM (○), and 1.6 mM (▼), or in the presence of lipase and in the absence of glycerol (G^-/L^+), with PD concentration being 0 mM (▽), 0.8 mM (■), and 1.6 mM (□). The reaction rate was determined as the rate for glycerol depletion.

can be applied in the estimation of lipase inhibition kinetics, only under the premise that the reactions proceeded in the condition of G^+/L^-, would yield identical results either with or without inhibitor.

All of kinetic results consistently indicate that PD competitively inhibited the pancreatic lipase activity. It acknowledges that PD might be directly bound to lipase/colipase. By now, only unspecific binding of saponins with cholesterol was postulated (Han *et al.*, 2002), and in the case of PD/PS, no direct experimental evidence was currently provided. Our findings make an important progress in illustrating how PD acts on pancreatic lipase to reduce triglyceride digestion and absorption.

Noticeably, although PD showed only a mild degree of lipase inhibition *in vitro*, the anti-obese effect shown *in vivo* was demonstrated at a significantly higher level (Burdette, 1986; Han, 2002). This suggests that PD involvement in the lipid metabolism of ameliorating obesity and hyperlipidemia is more profound than what lipase inhibition alone has done. Further studies are ongoing to illustrate its mediation pathways and to show to what extent it acts.

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