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Linoleic Acid from Bamboo (Phyllostachys Bambusoides) Displaying Potent α-Glucosidase Inhibition

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Glycosidase inhibitors are major targets in the treatment of type II diabetes, cancer and viral infections. This study was carried out to investigate the glycosidase inhibitory substances from bamboo (*Phyllostachys bambusoides*). Bamboo was extracted with methanol and then further fractionated with *n*-hexane, chloroform, *n*-BuOH and aqueous to get an active fraction. All extracts were evaluated for α-glucosidase inhibitory activities to identify the *n*-hexane fraction with 33.5 μg/ml of IC_{50} value. Active compound **1** in the *n*-hexane fraction was identified as linoleic acid, which exhibited inhibitory activity with 12.4 μM of IC_{50} value. Mechanistic analysis showed that linoleic acid exhibited non-competitive inhibition. This is the first study in which bamboo is reported to show α-glucosidase inhibitory activity.

Key words : Glycosidase, α-glucosidase inhibitor, *Phyllostachys bambusoides*, linoleic acid

Introduction

Screening of glycosidase inhibitors is becoming increasingly popular because they concern with treatment of numerous disease including diabetes mellitus type II [6], cancer [5], and HIV [13]. Glycosidase are involved in the biosynthesis and processing of oligosaccharide chains of N-linked glycoproteins in endoplasmic reticulum (ER). Inhibition of these glycosidases, especially α-glucosidase, has a profound effect on the glycan structure which consequently affects the maturation, transport, secretion, and function of glycoproteins, and could therefore alter cell-cell or cell-virus recognition processes [2,4,7]. For instance: by retarding the cleavage of complex carbohydrates, postprandial glucose absorption *in vivo* can be attenuated, thus regulating blood sugar levels in diabetics [12], the spread of cancer as well as the structural changes of cell surface glycoconjugates with in neoplasmic cells is proliferated by glycosidases in the sera and interstitial fluid around the tumor, thus by effecting glycosidase inhibition, cancer growth may be retarded [3]; finally, cellular signaling recognition is principally orchestrated by glycoproteins. α-Glucosidases (EC 3. 2. 1. 20; α-D-glucoside glucohydrolase) are a group of exo-acting enzymes that play essential roles in carbohydrate and quality control.

Bamboo is well renowned as a polyphenol plant that is one of the most ubiquitous traditional herbal medicines in East Asia. This species, belonging to the family of Gramineae, may be considered to be a nontoxic natural therapeutic agent. The main functional components are flavonoids, lactones, and phenolic acid in this species [9]. Its young leaves have been classified as edible by the KFDA (Korea Food & Drug Administration). Its young leave has also listed in the national standard (i.e. GB2760) as a kind of food antioxidant in china. Previous workers reported that the antioxidant product derived form bamboo leaves, was capable of blocking chain reaction of lipid autooxidation, chelating metal ions of transition state, and blocking the synthetic reaction of nitrosamine [11].

In the course of a continuing search for glycosidase inhibitor from plant source [8,10]. *n*-Hexane extract of bamboo were found to show significant α-glucosidase inhibitory activity. In this study, we isolated the target α-glucosidase inhibitor from the *n*-hexane extract of the leaves of bamboo and identified its structure using spectroscopic methods. Additionally, we carried out kinetic study for the isolated compounds.

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Materials and Methods

Plant material

The Bamboo (*Phyllostachys bambusoides*) were collected at Mt. cheongam-myeon in hadog Korea, on June 2008.

Extraction and isolation

The leaves (1 kg) of bamboo were air-dried and extracted with methanol (10 l×2) for 3 days at room temperature and filtered to remove the precipitate. The combined methanol extracts was concentrated in vacuum to yield a dark green gum (21 g). The methanol extract was dissolved in 1.5 l of mixture partitioned with *n*-hexane, $CHCl_3$, *n*-BuOH (each 3 l ×1), yielding *n*-hexane (4 g), $CHCl_3$ (7 g), *n*-BuOH (5 g), and H_2O extracts (4 g). The *n*-hexane phase was chromatographed on silica gel (3×30 cm, 230-400 mesh, 130 g) using *n*-hexane / EtOAc solvent system under gradient condition [20:1 (0.3 l), 10:1 (0.3 l), 5:1 (0.3 l), 2:1 (0.3 l)] to give 4 fractions. The fraction C (230 mg) was subjected to a reversed-phase column chromatography. Thus, this sample was loaded onto a glass column (1×50 cm), packed with RP-18 (ODS-A, 12 nm, S-150 μm, 40 g). The column was then eluted using MeOH:CH_3CN:H_2O (6:1:1) to afford compound 1 (95 mg). as an oily substance ; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, m, H-18), 1.33 (14H, m), 1.65 (2H, m), 2.05 (4H, m, H-8 and H-14), 2.36 (2H, m, H-2), 2.79 (2H, m, H-11), 5.37 (4H, m, H-9, 10, 12, and 13); ^{13}C NMR (75 MHz, $CDCl_3$) δ 180.2, 130.2, 130.0, 128.0, 127.8, 34.0, 31.5, 29.3, 29.0, 27.1, 25.6, 24.6, 22.5 and 14.0.

Enzyme assay

α-Glucosidase (EC 3.2.1.20, from Baker's), *p*-nitrophenyl-α-D-glucopyranoside used for the bioassay were purchased from Sigma Chemical Co. Nitrophenol Methods: The experimental procedure of Lianquan *et* al [14,15]. for the measurement of α-glucosidase activity was used with some modifications. The α-glucosidase activities were determined using an appropriate *p*-nitrophenyl-α-D-glucopyranoside as a substrate at the optimum pH of each enzyme. The reaction was stopped by adding 2 M NaOH. The released *p*-nitrophenol was measured Spectirometrically at 405 nm. The inhibitory effects of the tested compound were expressed as the concentration that inhibits 50% of the enzyme activity (IC_{50}). Kinetic parameters were determined by the Lineweaver-Burk double-reciprocal-plot method and Dixon plot method at increasing concentration of substrate and inhibitors.

Results and Discussion

Isolation and structural identification of target compound 1

For investigation of the glycosidase inhibitor, bamboo was extracted with methanol and then further fractionated with *n*-hexane, chloroform, *n*-buthanol and aqueous to get active extract. All extracts were tested for their enzymatic inhibitory activities against α-glucosidase from baker's yeast. The enzyme was assayed according to standard procedures by following the hydrolysis of nitrophenyl glycoside spectrophotometrically [14,15]. As shown in Table 1, *n*-hexane extract showed a significant degree (>95%) of α-glucosidase inhibition at 500 μg/ml sample concentration, while other extracts did not show.

As the concentrations of *n*-hexane extract increased, the residual enzyme activity rapidly diminished (Fig. 2A) Activity guided fraction of *n*-hexane extract gave compound 1 which was purified over octadecyl functionalized silica gel. The structural elucidation of compound 1 is detailed below. Compound 1 had the molecular formular $C_{18}H_{32}O_2$ and three degrees of unsaturation, as deduced from HREIMS (m/z 280.2402 [M^+]) data. The IR spectrum resembled those of fatty acid derivatives. The ^{1}H and ^{13}C NMR data including DEPT experiments showed the presence of eighteen carbon atoms: twelve methylene (sp^3), four methins (sp^2), one methyl, and one quaternary carbon. The presence of isolated methylene (H-11) was deduced from connectivity between methylene protons H-11 (δ_H 2.79, m) and methin protons (H-10 and H-12, δ_H 5.37, m) in the COSY spectrum. The COSY correlation between methylene protons (δ_H 2.05, 4H, m) and methins (H-9 and H-13, δ_H 5.37) was proved the presence of H-14 and H-8. Esterification of compound 1 with methanol yields methyllinolate that was ascertained by

Table 1. Inhibition percentage of α-glucosidase activity of extracts from *Phyllostachys bambusoides*

Fraction	Inhibition (%)[a)]	IC_{50} μg/ml
n-Hexane	>95%	33.6
$CHCl_3$	40%	NI[b)]
MeOH	35%	NI[b)]
Water	NI[b)]	NI[b)]

[a)]All tests were run at 500 μg/ml sample concentration, values are means of three experiments [b)]NI means no inhibition.

Table 2. α-glucosidase assay results for compound 1

Compound	IC_{50}[a] (μM)	K_i value	K_m
1	12.4	9.89	301.41
Voglibose	23.4	NT[b]	NT[b]

a)Compound were examined in a set of experiments repeated three times; IC_{50} values of compound represent the concentration that caused 50% enzyme activity loss. [b]NT means not test.

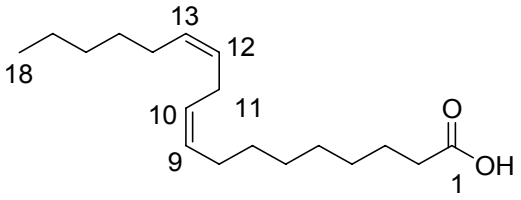

Fig. 1. Structure of compound 1.

GC/MS analysis with reference to an authentic sample. Thus, compound 1 was identified as linoleic acid (Fig. 1).

α-Glucosidase inhibitory activity and kinetic analysis

Isolated compound 1 showed dose-dependent effect on α-glucosidase activity (Fig. 2A). The inhibitory potencies and capacities of compound 1 towards α-glucosidase activity was evaluated as 12.4 μM of IC_{50} value. The potency of linoleic acid (IC_{50} 12.4 μM) copares with sugar-derived α-glucosidase inhibitor currently used for therapeutic purpose such as voglibose (IC_{50} 23.4 μM) [1]. The inhibition mechanisms displayed by the isolated compound 1 were subsequently studied. The compound 1 manifested the same relationship between enzyme activity and enzyme concentration. The inhibition of α-glucosidase by compound 1 is illustrated (Fig. 2B). Plots of the initial velocity verus enzyme concentration in the presence of different concentration of compound 1 gave a family of straight lines, all of which passed through the origin. Increasing the inhibitor concentration resulted in a lowering of the line gradients, indicating that the compound was reversible inhibitor.

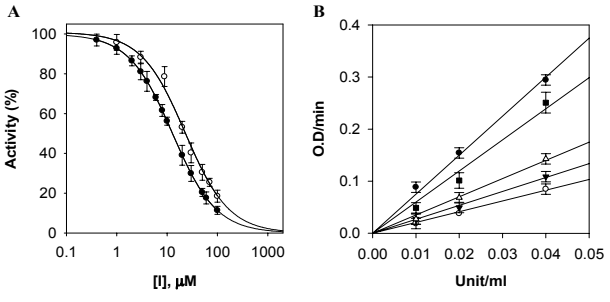

Fig. 2. (A) Effect of n-hexane fraction (◯) and compound 1 (●) on the activity of α-glucosidase for the hydrolysis of *p*-nitirophenyl-α-D-glucopyranoside. (B) Relationship of the hydrolytic activity of α-glucosidase with enzyme concentrations at different concentrations of compound 1. Concentration of compound 1 for cure from top to bottom: 8.9, 17.8, 35.6 and 71.3 μM.

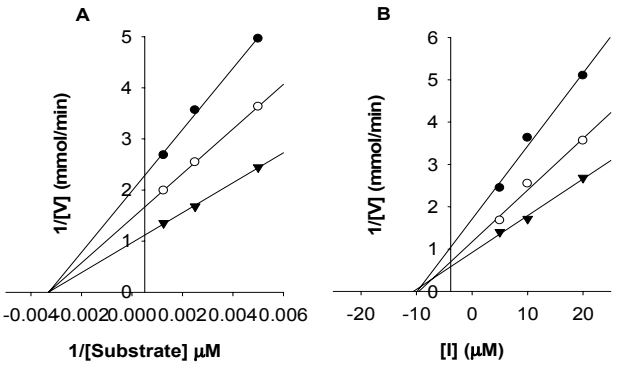

Fig. 3. Graphical determination of the type of inhibition for compound 1. (A) Lineweaver-Bukr plot for inhibition of compound 1 on α-glucosidase for the hydrolysis of *p*-nitirophenyl-α-D-glucopyranoside. In the presence of different concentration of compound were 17.8 (▼), 35.6 (◯) and 71.3 μM (●), respectively. (B) Dixon plot; inset is replot of *slope* versus the corresponding 1/[S] of compound 1.

Finally, we investigated the characteristics of inhibitor 1 with respect to the two different steps carried out by the enzyme individually. In this experiment, the initial velocity of the enzyme was monitored by observing nitrophenol formation at 405 nm. The enzyme inhibition properties of compound 1 were modeled using double reciprocal plot (Lineweaver-Burk plot). The results showed that V_{max} value decreased without change in K_m value in the presence of inhibitor (Fig. 3A). Therefore, $-1/K_m$, the x - intercept stayed the same, and $1/V_{max}$ get more positive. Therefore, compound 1 exhibit noncompetitive inhibition model for α-glucosidase. As shown Fig. 3B, K_i value of compound 1 was estimated as 9.9 μM from Dixon plots (Fig. 3B).

In conclusion, we have proven that hexane extract from the leaves of bamboo possess highly potent α-glucosidase inhibition properties. Interestingly, effective component was identified as linoleic acid displaying an IC_{50} value of 12.4 μM. This is the first report that bamboo posses a α-glucosidase inhibitory activity.

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초록 : 대나무로부터 분리한 linoleic acid의 α-glucosidase 저해활성 연구

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당가수분해효소 저해제는 2형 당뇨병, 암, 바이러스 감염제 개발의 주요 타켓화합물이 되고 있다. 본 연구에서는 기능성식품 소재로 이용될 수 있는 대나무 잎에서 α-glucosidase 저해제를 탐색하였다. 대나무 잎을 메탄올 용매로 추출하고, 이들을 핵산, 클로로포름, 부탄올로 용매분획 하였다. 용매 분획된 각 추출물의 α-glucosidase 저해활성을 검정시험에서 핵산 분획층에서 강한 α-glucosidase 저해활성(IC_{50} 33.5 μg/ml)을 관찰하였다. 높은 저해활성을 보여준 핵산 분획층의 활성물질은 linoleic acid로 구조동정 되었다. 분리된 linoleic acid는 IC_{50}이 12.4 μM로 높은 α-glucosidase 저해활성을 나타내었다. 저해활성 메커니즘 연구에서 linoleic acid는 비경쟁적 저해 양상을 나타내었다. 본 연구는 대나무가 α-glucosidase 저해활성을 나타내는 첫 번째 연구 결과이다.