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Anti-diabetic activities of phenolic compounds in muscadine against alpha-glucosidase and pancreatic lipase

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ABSTRACT

Inhibitory mode and anti-diabetic activities of the Carols muscadine extracts on the α-glucosidase and pancreatic lipase were investigated. The IC_{50} values of the muscadine and its seed methanolic extracts against the α-glucosidase were 1.92 and 1.53 mg/mL, and those against the lipase were 34.41 and 8.63 mg/mL, respectively, which indicated the muscadine possessed the strong anti-diabetes activity. Particularly, the ethyl acetate (EtOAc) extract fractions exhibited the highest inhibitory activities against both enzymes. Since most of the phenolic compounds existed in the EtOAc fractions, it is suggested that phenolic compounds may be the main source against the α-glucosidase and pancreatic lipase. Twelve individual phenolics were identified by HPLC-MS. Ellagic acid and quercetin exhibited much stronger anti-diabetic activities among the selected phenolic standards. Besides, the extracts and the phenolic standards obeyed the competitive mode in their enzymatic inhibitions.

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1. Introduction

Muscadine (*Vitis rotundifolia*) is a grape vine species native to the present-day southeastern United States, where it has been extensively cultivated since the 16th century. The fruit contains a high amount of polyphenols and other nutrients that make it the latest subject for health-benefiting studies. The identified phytochemicals in muscadine grapes included phenolic acids, flavonoids and anthocyanins. In the last several decades, phenolic compounds have attracted much attention due to their various biological activities, such as anti-inflammatory activity, estrogenic activity, enzyme inhibition, anti-microbial activity (including anti-fungal and anti-bacterial activity) (Cushnie & Lamb, 2005), anti-allergic activity, antioxidant activity, vascular activity and cytotoxic anti-tumor activity (Harborne & Williams, 2000), anti-cancer properties (McCann et al., 2007), etc.

Anti-diabetic property of phenolics, especially from grapes, has been recently studied (Montaguta et al., 2010). Diabetes mellitus, also simply referred to as diabetes, is a metabolic diseases characterized with a high blood glucose level that can cause serious damage to body system, such as blood vessels and nerves (Matsui et al., 2007). There are three main types of diabetes, type I, type II and gestational diabetes. Type II diabetes is the most common, which is affecting 90–95% of the U.S. diabetes population (Wild, Roglic, Green, Sicree, & King, 2004).

One of the therapeutic approaches to treat the diabetes is to decrease the postprandial hyperglycemia by retarding absorption of glucose. Inhibition of carbohydrate-hydrolyzing enzymes, such as α-glucosidase, is considered a possible pathway because the enzyme plays a key role in digesting carbohydrates (Bhandari, Nilubon, Gao, & Kawabata, 2008; Krentz & Bailey, 2005). On the other side, the type I diabetes is caused by progressive destruction of pancreatic insulin-producing β cells, which could be damaged by the accumulated lipids in the pancreas. Therefore, lipase inhibitors have attracted much attention in order to reduce the lipid absorption for their anti-obesity activities, and protect the pancreas that will enable the β cells to produce normal level of insulin.

So far, study of the anti-diabetic property of muscadine is scarce. Therefore, the aims of this study were to (1) investigate the anti-diabetic activities of muscadine extract fractions in different solvents; (2) explore the inhibitive mode and enzymatic parameters such as accurate dissociation constant (K_i) and IC_{50} values on the α-glucosidase and pancreatic lipase; and (3) characterize the functional phenolic compounds by high performance liquid chromatography with mass spectrometry (HPLC-MS).

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2. Materials and methods

2.1. Materials

Standards such as gallic acid, protocatechuic acid, p-coumaric acid, catechin, epicatechin, ellagic acid, myricetin, quercetin, resveratrol, were obtained from Sigma–Aldrich (St. Louis, MO). Alpha-glucosidase (EC 3.2.1.20) from *Saccharomyces cervisiae*, lipase (EC 3.1.1.3) from porcine pancreas type II, ρ-nitrophenyl-α-D-glucopyranoside, and 4-methylumbelliferyl oleate, were purchased from Sigma–Aldrich (St. Louis, MO). Tris, hydrochloric acid (HCl), acetic acid, HPLC grade methanol and acetonitrile were from Fisher Scientific (Suwannee, GA). Chloroform, ethyl acetate, and n-butanol of analytical grade were purchased from Fisher Scientific (Suwannee, GA) as well.

2.2. Sample collection and preparation

Carols muscadine from Paulk Vineyard (Wray, GA) is a vigorous, productive, self-fertile cultivar in Southern US regions. The muscadine seed was also provided by the Paulk Vineyard. All samples were stored at −20 °C until analysis. Phenolic compounds from 20 g of the pre-grounded Carols muscadine and its seed were extracted with 400 mL (mL) of a mixture of methanol (MeOH): water in the ratio of 80:20 and with 0.1 mL/L acetic acid, accompanied by 1 h of sonic treatment. Then, the extract was transferred into a 500 mL bottle through a 0.45 μm cellulose acetate filter (Costar Corp. Cambridge, MA) to prepare the original methanolic whole extracts. The extracts were concentrated equivalent to 1 g fresh material/mL.

To prepare other extracts, 10 mL of the methanolic extracts were concentrated to remove the solvent completely with a vacuum rotary evaporator, and then suspended in 100 mL distilled water. Then the methanolic extracts dissolved in water were sequentially fractionated by chloroform ($CHCl_3$), ethyl acetate (EtOAc), and n-butanol (BuOH), using liquid–liquid extraction (Zhang, Wang, Chen, Androulakis, & Wargovich, 2007). The remaining part after three solvent extractions was the H_2O extract. All the extracts were concentrated by the vacuum rotary evaporator to remove the solvent completely, and redissolved in methanol again. The analyzed concentration for all 4 fractionated extracts (i.e. the $CHCl_3$ extract, EtOAc extract, BuOH extract, and H_2O extract) was accurately weighed equivalent to 2 g fresh material/mL.

2.3. Alpha-glucosidase inhibitory activity

All samples, the Carols muscadine whole fruit methanolic extract (CME), Carols $CHCl_3$ extract (CCE), Carols EtoAc extract (CEE), Carols BuOH extract (CBE), Carols water extract (CWE), and the seed methanolic extract (SME), the seed $CHCl_3$ extract (SCE), the seed EtoAc extract (SEE), the seed BuOH extract (SBE), and the seed water extract (SWE) were assayed by the α-glucosidase. This bioassay was modified from the Suresh's study using ρ-nitrophenyl-α-D-glucopyranoside (ρNPG) as the substrate, which is hydrolyzed by α-glucosidase to release ρ-nitrophenol, a color agent that can be monitored at 405 nm (Suresh et al., 2004). Since α-glucosidase is sensitive to different pH values, the enzyme was prepared with 0.1 mol/L phosphate buffer (pH 6.8). An aliquot of 20 μL of the sample solution was mixed with 70 μL of the enzyme solution (1 unit/mL) and incubated at 37 °C for 6 min under shaking. The blank used methanol instead of the sample. After incubation, 100 μL of 4 mmol/L ρNPG solution in the above buffer solution (pH 6.8) was added to initiate the colorimetric reaction at 37 °C. The released ρ-nitrophenol from ρNPG was monitored immediately at 405 nm every min for 60 min by a BioTek μQuant 96 micro well plate reader (Bio-Tek® Instruments, Inc., Winooski, VT). The α-glucosidase inhibition activity was determined by measuring the effect on the enzyme reaction rate after adding Muscadine extracts, compared with the control.

2.4. Pancreatic lipase inhibitory activity

The method was modified from the assay reported by Nakai et al. (2005), in which 4-methylumbelliferyl oleate (4-MU oleate) was used as a substrate to measure the pancreatic lipase inhibitory activity of all samples. Briefly, the assay was conducted by mixing 50 μL of the pancreatic lipase solution (2 unit/mL) in a buffer consisting of 50 mmol/L Tris–HCl (pH 8.0), 100 μL of diluted sample solutions and 50 μL of 0.5 mmol/L 4-MU solution dissolved in the above buffer in the well of a 96 micro well plate to start the enzyme reaction. The plate was immediately placed in the 37 °C pre-heating FL × 800 micro plate fluorescence reader (Bio-Tek® Instruments, Inc., Winooski, VT) to measure the amount of 4-methylumbelliferone released by lipase every minute for 30 min at an excitation wavelength of 360 nm with a tolerance of ±40 nm and an emission wavelength of 455 nm with a tolerance of ±20 nm. As same as the α-glucosidase inhibition measurement, the lipase inhibitive activity was determined by measuring the effect on the enzyme reaction rate after adding extracts, compared with the control.

2.5. Determine the inhibition mode and the K_i and IC_{50} values of α-glucosidase and pancreatic lipase inhibitory activities

To determine the accurate K_i and IC_{50} values of all samples, the V_{max} and K_m constants need to be determined at first using the Lineweaver–Burk plots from the relevant Michaelis–Menten equations. The ρNPG substrate solutions in concentrations of 1, 2, 3, 4, and 5 mmol/L for the α-glucosidase inhibition assay and the 4-MU solutions in concentrations of 0.05, 0.1, 0.25, and 0.5 mmol/L for the pancreatic lipase inhibitory activity were reacted with methanol as control. Meanwhile, the same substrate solutions were used for the Carols methanolic whole extracts, the seed methanolic extracts, and standards of catechin, quercetin and ellagic acid to determine their appropriate inhibition modes against the enzymes. Then the K_i values of the test samples were obtained from the least-square regression lines of the plots of the reciprocal of the sample (inhibitor) concentration versus the reciprocal of the rate of reactions, while the concentration of 4 mmol/L was chosen for the α-glucosidase inhibitory activity and 0.5 mmol/L for the lipase inhibition assay, for which the formula is listed below:

$$\frac{1}{V} = \frac{K_m}{V_{max}K_i[S]}[I] + \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right)$$

The IC_{50} value was obtained from:

$$IC_{50} = K_i\left(1 + \frac{[S]}{K_m}\right)$$

Where the K_m is the Michaelis constant and the K_i is the dissociation constant. The V_{max} is the maximum rate of the enzymatic reaction. The $[S]$ represents the concentration of substrate and $[I]$ is the concentration of sample (inhibitor) solution. The IC_{50} value is the concentration of sample (inhibitor) to provide 50% inhibitory activity.

2.6. Total phenolic content

The total phenolic content (TPC) was determined by a modified Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Briefly, aliquots of 100 μL of the standard chemical gallic acid