Bioassay-Guided Separation of an α-Amylase Inhibitor Anthocyanin from *Vaccinium arctostaphylos* Berries

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Z. Naturforsch. **65c**, 567–570 (2010); received March 28/June 24, 2010

Vaccinium arctostaphylos is a traditional medicinal plant in Iran used for the treatment of diabetes mellitus. In our search for antidiabetic compounds from natural sources, we found that the extract obtained from *V. arctostaphylos* berries showed an inhibitory effect on pancreatic α-amylase *in vitro* [IC_{50} = 1.91 (1.89–1.94) mg/mL]. The activity-guided purification of the extract led to the isolation of malvidin-3-*O*-β-glucoside as an α-amylase inhibitor. The compound demonstrated a dose-dependent enzyme inihibitory activity [IC_{50} = 0.329 (0.316–0.342) mM].

Key words: Vaccinium arctostaphylos, α-Amylase Inhibitory Activity, Malvidin-3-*O*-β-glucoside

Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels. One goal of therapy for diabetic patients, especially non-insulin-dependent diabetes mellitus (type 2 diabetes), is the maintenance of normal blood glucose levels after a meal (postprandial hyperglycemia) (Li *et al*., 2005; Mai and Chuyen, 2007). A therapeutic approach for decreasing blood glucose level after a meal is to retard the absorption of glucose by inhibition of carbohydrate-hydrolyzing enzymes, such as α-amylase and α-glucosidases. The inhibition of the enzymes significantly decreases the digestion and absorption of carbohydrates, thereby decreasing the postprandial blood glucose levels (Ali *et al*., 2006; Dewi *et al*., 2007; Kwon *et al*., 2006). Thus, there is a need to develop compounds with enzyme inhibitory activities.

The genus *Vaccinium* (Ericaceae) comprises nearly 200 species, most of them found in the northern hemisphere (Morazzoni and Bombardelli, 1996). The genus is represented in Iran only by the species *Vaccinium arctostaphylos* L. (Wendelbo, 1965). *V. arctostaphylos* is a shrub found in the northern forests of Iran between 1600 and 1800 m above sea level and is locally known as "Qaraqat" and/or "Cyah-gileh". *V. arctostaphylos* is used traditionally as food and for its medicinal values. In traditional Iranian medicine, the decoction from the berries has been used as antidiabetic and antihypertensive agent for a long time (Amin, 1991; Ghahreman, 2001).

Different parts of *V. arctostaphylos* have been reported to exhibit various biological activities, such as antioxidant and antimicrobial effects (Tosun *et al*., 2004; Taherpour *et al*., 2008; Koca and Karadeniz, 2009). However, there is no report on an antihyperglycemic activity and α-amylase inhibitory effect of *V. arctostaphylos* berries. A bioactivity-guided study of the extract obtained from *V. arctostaphylos* berries was performed to investigate the α-amylase inhibitory activity of the extract and to isolate the compound responsible for the inhibitory activity.

Material and Methods

Plant material

The ripe berries of *V. arctostaphylos* were collected from the forest region of Asalem in the north of Iran in August 2002. Voucher specimens were deposited in the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (no. 6520 THE).

Extraction, chromatography, and spectroscopy

The berries of the plant were air-dried at room temperature, pulverized, and extracted by mac-

0939–5075/2010/0900–0567 $ 06.00

eration of 400 g of the berries with methanol/glacial acetic acid/water (70:2:28 v/v/v). The extract was filtered, concentrated, and then partitioned against ethyl acetate. The aqueous part was fractionated by repeated descending preparative paper chromatography (PC) on Whatman No. 3MM paper using *n*-butanol/glacial acetic acid/water (4:1:5 v/v/v, upper phase) (BAW) and 15% aqueous acetic acid (15% HOAc) as developing solvents. The bands were eluted using methanol/glacial acetic acid/water (90:5:5 v/v/v), the eluent concentrated, and monitored by descending PC on Whatman No. 1 paper (Glassgen *et al*., 1992).

The pure compound was identified on the basis of spectral and chromatographic studies. The UV-Vis spectrum was recorded on a Shimadzu UV-160A spectrophotometer in 0.01 M HCl/CH_3OH; the ^1H NMR spectrum was taken on a Bruker Spectrospin 500 spectrometer in CD_3OD/CF_3COOD (5:1), and chemical shifts were recorded as δ values; the positive FAB-MS spectrum was obtained in glycerol/HCl as the matrix by a Finnigan MAT TSQ 700 mass spectrometer. The nature of the sugar moiety in the isolated compound was determined through acid hydrolysis, according to standard methods (Francis *et al*., 1966) and direct comparison of the moiety with authentic carbohydrates by co-chromatography on Merck cellulose TLC plates.

α-Amylase inhibition test

The α-amylase inhibitory activity was determined using the method described previously (Nickavar *et al*., 2008). Briefly, 1 mL of the porcine pancreatic α-amylase enzyme solution (0.5 IU/mL) in 20 mM phosphate buffer (pH 6.9) was incubated with 1 mL of each test (at various concentrations) for 30 min. The reaction was initiated by adding 1 mL of 0.5% soluble potato starch solution, and the mixture was incubated for 3 min at 25 °C. Then, 1 mL of the colour reagent (96 mM 3,5-dinitrosalicylic acid and 5.31 M sodium potassium tartrate in 2 M sodium hydroxide) was added, and the mixture was placed in a water bath at 85 °C. After 15 min, the reaction mixture was diluted with distilled water, and the absorbance determined at 540 nm. Individual blanks were prepared for correcting the background absorbance. In this case, the colour reagent solution was added prior to the addition of starch solution and the mixture then placed in the water bath immediately. Controls were representative of the 100% enzyme activity. They were conducted in an identical fashion replacing tests with 1 mL of the solvent. Acarbose, a well-known α-amylase inhibitor, was used as positive control. The inhibition percentage of α-amylase was assessed by the following formula:

$$I_{\alpha\text{-amylase}}(\%) = 100 \cdot \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right),$$

where A_{control} is the absorbance of each control, and A_{sample} is the net absorbance of each sample. The net absorbance of each sample was calculated as:

$$A_{\text{sample}} = A_{\text{test}} - A_{\text{blank}},$$

where A_{test} is the absorbance of the test, and A_{blank} is the absorbance of each blank.

The $I_{\alpha\text{-amylase}}(\%)$ for each sample was plotted against the logarithm of sample concentration, and a logarithmic regression curve was established in order to calculate the IC_{50} value. The assays were performed in triplicate.

Malvidin-3-O-β-glucoside

UV-Vis: λ_{max} (CH_3OH/HCl) = 539, 277.5, 215.5 nm; +$AlCl_3$ (5% $AlCl_3$ in CH_3OH): 539, 277.5, 215.5 nm. – $E_{440}/E_{vis,max}$ = 23%. – ^1H NMR (500 MHz, CD_3OD/CF_3COOD): δ (aglycone moiety) = 9.03 (1H, s, H-4), 7.97 (2H, s, H-2' and H-6'), 6.94 (1H, d, J = 1.5 Hz, H-8), 6.66 (1H, d, J = 1.5 Hz, H-6), 3.99 (6H, s, OCH_3-3' and OCH_3-5'); δ (sugar moiety) = 5.25 (1H, d, J = 7.6 Hz, H-1"), 3.84–3.30 (5H, m, H-2"–H-6"). – FAB-MS: m/z = 493 for $[M^+] = C_{23}H_{25}O_{12}^+$.

Results and Discussion

The extract obtained from *V. arctostaphylos* berries produced a dose-dependent reduction in the α-amylase activity [IC_{50} = 1.91 (1.89–1.94) mg/mL] (Table I).

The PC analysis of the aqueous part revealed at least three major compounds. The most active compound of the aqueous part involved in the inhibition of the enzyme had R_f = 0.32 and R_f = 0.49 with BAW and 15% HOAc, respectively. The chromatographic and spectral data of the compound revealed that it was malvidin-3-*O*-β-glucoside (Mv-3-glc) (Fig. 1), and all of its data matched with those reported in the literature (Cabrita and Andersen, 1999). The compound

Table I. α-Amylase inhibitory activities and IC_{50} values of the berry extract of *Vaccinium arctostaphylos* and its active compound Mv-3-glc.

Concentration	Inhibition (%)[a]	IC_{50}[b]
Berry extract [mg/mL]		
3.6	84.95 ± 0.94	
2.88	74. 97 ± 0.98	
2.30	60.96 ± 0.46	1.91 (1.89–1.94) mg/mL
1.84	36.66 ± 0.79	
1.47	19.48 ± 0.53	
Mv-3-glc [mM]		
0.913	72.60 ± 0.67	
0.584	55.15 ± 0.67	
0.374	44.05 ± 0.90	0.329 (0.316–0.342) mM
0.239	21.79 ± 0.65	
0.153	8.75 ± 0.66	

[a] The data are expressed as means ± SEM of five experiments in each group.
[b] The IC_{50} values were established by logarithmic regression curves with normalized data (using the computer software GraphPad Prism 3.02 for Windows) and are presented as their respective 95% confidence limits.

inhibited α-amylase activity in a dose-dependent manner. The IC_{50} value was 0.329 (0.316–0.342) mM (Table I). However, the data indicate that malvidin-3-*O*-β-glucoside is not as potent as the reference inhibitor acarbose [IC_{50} = 0.033 (0.031–0.036) mM].

Generally, the anthocyanins of the genus *Vaccinium* have received much attention due to their relevant pharmacological properties. Malvidin-3-*O*-β-glucoside has already been isolated from some species belonging to the genus including *V. arctostaphylos* (Ballinger *et al*., 1981, 1982; Baj *et al*., 1983; Andersen, 1985, 1987a, b, 1989; Gao and Mazza, 1994; Cabrita and Andersen, 1999; Nickavar and Amin, 2004; Grace *et al*. 2009). However, to the best of our knowledge, this is the first report on the inhibitory effect of malvidin-3-*O*-β-glucoside on α-amylase.

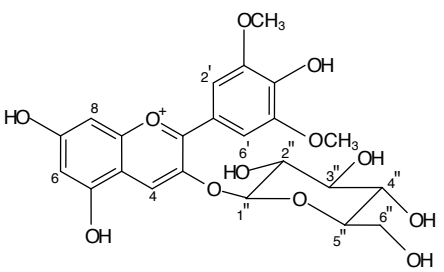

Fig. 1. Chemical structure of malvidin-3-*O*-β-glucoside.

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