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UHPLC/MS Identifying Potent α -glucosidase Inhibitors of Grape Pomace via Enzyme Immobilized Method

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Abstract

α -Glucosidases have been a major target in controlling and managing postprandial blood glucose and therefore diabetes treatment. This study aims to further identify and purify active compounds from the most active ethyl acetate fraction collected previously in Tinta Cão grape pomace extract (TCEE) using a newly developed and highly effective immobilization method, including obtaining compounds previously shown to inhibit the enzyme. Purification used crosslinked chitosan beads with α -glucosidases bound to polymer, which acted as immobilized enzyme vehicle to collect inhibitors. Compounds absorbed into the beads were eluted using methanol, where collected fraction was subjected to UHPLC-MS analysis to identify active compounds. Results presented 5 major compounds: viniferifuran (amurensin H), *p*-coumaroyl-6-*O*-*D*-glucopyranoside, *p*-coumaroyl-6-*O*-hexoside, (epi)catechin-hexoside, 10-carboxyl-pyranopeonidin 3-*O*-(6''-*O*-*p*-coumaroyl)-glucoside. These findings indicated the particular molecules can be utilized as potent α -glucosidases inhibitors, and may be further tested for postprandial glucose control.

Keywords

Chitosan beads; α -glucosidase inhibitor; immobilization; Tinta Cão grape pomace; UHPLC/MS

Introduction

The identification and purification of natural compounds with improving methodologies is one of the major areas being continuously researched. Currently, one main target for managing postprandial hyperglycemia and treatment of T2DM is to search for α -glucosidase inhibitory agents that significantly decrease carbohydrate digestion thus lowering glucose uptake (DiNicolantonio, Bhutani, & O'Keefe, 2015; Inzucchi et al., 2012; Van de Laar, Lucassen, Akkermans, Van de Lisdonk, & De Grauw, 2006). After we found that grape pomace extract suppressed postprandial hyperglycemia in diabetic mice by specifically inhibiting α -glucosidase (Hogan et al., 2010), a pair of anomers of 6-*O*-*p*-trans-coumaroyl-

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Conflict of Interest

The authors have no conflict of interest.

Author Contributions

Kequan Zhou designed the study and revised the paper. Wenjun Zhu performed enzyme stabilization and inhibition test, Shi Sun performed sample preparation and analysis; Wenjun Zhu and Shi Sun wrote the manuscript. Fei Yang helped to conduct data analyses.

D-glucopyranosides, were identified from Tinta Cão Grape Pomace using a bioassay guided fractionation method in combination with HPLC and NMR analysis. The compound was isolated from the strongest α -glucosidase inhibition fraction obtained through ethyl acetate soluble portion of the pomace extract (TCEE) (Sun, Kadouh, Zhu, & Zhou, 2016). During the process, preliminary results suggested other potential inhibitory compounds might also presented in such fraction that were planned to be isolated and purified following the study. In addition, it was an objective to search purification method that might increase the yield and purity of the inhibitory compounds from the TCEE and possibly other fractions. The previous HPLC method and bioactive-guided assay did not efficiently work to supply more potent α -glucosidase inhibitor from TCEE (Kadouh, Sun, Zhu, & Zhou, 2016; Sun et al., 2016). As a long-term project for exploring potent antihyperglycemia agent from grape pomace, it is necessary to set up an efficient assay and analytical method for α -glucosidase inhibitors.

In order to improve the efficiency of separation and purification, it was presented that biological fractionation method has become increasingly effective in researching enzyme specific inhibitors. In particular, enzyme immobilization technique utilizing derived and cross-linked polymer was frequently used in many studies in order to purify natural compounds for the identification of specific molecules (Akku, Scedil, Cetinus, & Oztop, 2000). Furthermore, the method such as using polymerized chitosan beads to immobilize enzymes has been served as a great vehicle for conserve valuable enzymes and achieved reusability in biological interactions for elucidation of potential inhibitors (Basak & Aydemir, 2013; Deng, Xia, & Xiao, 2014; Dincer, Okutucu, Zihnioglu, & Telefoncu, 2005). The simple preparation process and sample absorption including other functions such as filtration, and so on made chitosan beads increasingly widely applied in biomedical and food industry.

This study was to prepare chitosan beads immobilized with intestinal α -glucosidases, and next to isolate and purify additional inhibitors from previously found TCEE fraction. The aim was to improve separation efficacy and increase the yield of potential inhibitors.

Materials and Methods

Plant material

Fresh Tinta Cão (*Vitis vinifera*) grape pomace was provided by Chrysalis Vineyards (Middleburg, VA, U.S.A.) via the Agricultural Research Station at Virginia State Univ. (Petersburg, VA, U.S.A.).

Chemicals and reagent

Chitosan (medium molecular weight), sodium hydroxide, citric acid, sodium phosphate monohydrate and rat intestinal acetone powder and 4-nitrophenyl- α -D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). 3% glutaraldehyde was obtained from Ricca Chemical Co. (Arlington, TX, U.S.A.). Acarbose was purchased from LKT, Inc. (St. Paul, MN, U.S.A.).

Chitosan beads

Chitosan solution was prepared from 2% w/v powder dissolved in 5% v/v acetic acid solution and dropped into 2 M NaOH (20% v/v EtOH) solution while stirring gently with a syringe through a 20 gauge needle. Chitosan beads formed instantly in strong base and product diameter is average 3 to 4 mm. After washing with deionized water until washing solution reached neutral pH, beads were collected for absorption or stored in DI water for later use.

Cross-linking and immobilization

To process absorption, 35 mL of freshly made Chitosan beads were measured by volume and placed in 40 mL of 3% glutaraldehyde buffer for cross-linking. Beads were incubated with shaking at 150 rpm under room temperature (25 °C) for 4 hr. After washing thrice with 0.1 M phosphate citric acid buffer (PCB) pH 5.0, 40 mL of α -glucosidase solution (previously described) (Sun et al., 2016) at 2.5 mg/mL was used to submerge the beads and shaken for 4 hr at 37 °C 150 rpm. Again beads were rinsed thrice with PCB and crosslinking-absorption procedure was repeated to maximize enzyme immobilization.

Sample absorption

Following PCB wash, chitosan beads were incubated and shaken in 40 mL of 2.5 mg/mL TCEE at 37 °C 150 rpm for 5 hr. Potential inhibitor(s) were attached onto beads with enzyme. After washed thrice with PCB, approximately 40 mL of 100% MeOH split into 3 portions were used to elute inhibitors through a filtration flask. Combined eluates were dried using Buchi R-124 rotary-evaporator. Fraction containing potential inhibitors was placed in scintillation vial and dried under nitrogen stream.

Sample enzymatic and inhibitory activities

During cross-linking absorption, before and after enzyme solutions including TCEE solutions were collected to test immobilization and enzyme inhibitory activity changes. Colorimetric assay for 96-well micro plate was used to assess α -glucosidase activities with Perkin Elmer HTS 7000 bio assay Reader. At 405 nm, 45 μ L enzyme solutions from before and after cross-linking absorptions were tested using 22.5 μ L p-NPG reagent as substrate and compared for differences in activities. 58 μ L of sample eluates at 2.5 mg/mL were analyzed using the same methods as above and compared to acarbose at 50 μ g/mL served as positive control. Resulting data was compared against TCEE activities to evaluate purification efficiency. Data was expressed in percentage inhibition in relation to acarbose using following formula for calculations.

$$\% \text{ Inhibition} = 100 - \left[\left(\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}} \right) \times 100 \right]$$

UHPLC/MS analysis

UHPLC-MS was performed on a Shimadzu TQ LCMS-8040, Nexera LC System (Shimadzu Corp., Japan). Analytes were separated on an ACQUITY UPLC HSS T3 Column (2.1 mm $\times$ 50 mm, 100Å, 1.8 μ m, Waters Corp., MA). The solvent system consisted of solvent A (0.1%

formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile, v/v). The solvent B content of the mobile phase was maintained at 5% in 0.05 min and then gradient increased to 35% in 6.50 min, 80% in 7.50 min, and keep for 1.1 min. The flow rate was held constant at 0.60 mL/min at 40 °C. Injection volume was 5 μ L.

Mass analysis was performed in both of positive and negative ESI ion full scan mode. The operating parameters were as follows: Nebulizing gas flow, 3 L/min, drying gas (N₂) flow rate, 15 L/min; DL temperature, 250 °C; heat block temperature 400 °C. The mass range was set at m/z 100 to 2000. The system was operated under LabSolution workstation acquisition software.

Results and Discussion

α -Glucosidase activity

Beads formation and cross-linking-absorption procedures were adopted from Akku et al. (2000) and Zhou, Wang, Wu, Tang, and Pan (2013). The assurance of immobilization was verified by testing α -glucosidase solution used for bonding to chitosan beads. Based on suggested methods, twice cross-linking-absorption was done to maximizing immobilization efficiency (Zhou et al., 2013). α -Glucosidase solution was evaluated for activities listed in Figure 1A. The percentage activity of initial and after immobilization were $68.08\% \pm 1.94\%$ and $15.26\% \pm 1.96\%$, respectively.

Extract and eluate inhibitory activity

TCEE sample was determined to have significant α -glucosidase inhibition level compared to control sample, Acarbose at 50 μ g/mL (Sun et al., 2016). Figure 1B illustrated the percentage inhibition of TCEE samples before and after attachment, which were $49.34\% \pm 2.77\%$ and $33.16\% \pm 0.91\%$, respectively. The eluates were also evaluated to possess a significant increase in inhibition level of $77.28\% \pm 3.04\%$, which indicated the successful stripping of the active inhibitors. Thus within the purified sub-fraction from TCEE eluate, it could be further analyzed for specific compositions and structure of molecular compounds. Based on the findings, TCEE solution used for absorption showed no significant loss in inhibition activity, suggesting there might be other inhibitors present in the fraction but unable to be attached onto immobilized α -glucosidases. There also might be a factor of affinity differences between strong and weak inhibitors. Due to the competitive nature of some inhibitors, high affinity level might associated with those that could be eluted later into final fractions; those with lower level remained in TCEE absorption solution.

UHPLC/MS analysis

After UHPLC/MS analysis of the eluates, 5 compounds bound to α -glucosidase were identified and the detailed chemical information was listed in Table 1 and Figure 2.

In the MS/MS spectrum of compound **1**, quasimolecular ions of m/z 453 [M+H] and 475 [M+Na] formed multiple product ions at m/z 367, 359, 254, 249, and 227. The ion at m/z 357 is generated from the loss of a single phenol group (94 Da), at m/z 249 from the successive loss of the second phenol group (110 Da). 42 Da (C₂H₂O), 94 Da (C₆H₆O), 106 Da

(C₇H₆O), and 110 Da (C₆H₆O₂) is characteristic neutral losses of stilbenoid dimers fragmentation (Moss, Mao, Taylor, & Saucier, 2013). However, the molecular weights of compound **1** is lower 2Da than common resveratrol dimers', 454, inclusive of trans/cis- ϵ/δ -viniferin, pallidol, parthenocissin A, and quadrangularin A that have been previously reported in grape root and stem or wine (Moss et al., 2013; Pawlus, Waffo-Teguo, Shaver, & Merillon, 2012). In addition, the most abundant ion at m/z 227 is generated from 2 dehydro-resveratrol moieties (226 Da) by cleavage in positive mode (Figure 3). Dependent on the fragmentation pattern, this compound was identified as viniferifuran, 1 viniferin-type stilbenoid dimer with a benzofuran ring system, which ever isolated from the stem of *V. vinifera* (Ito, Takaya, Oshima, & Niwa, 1999) and from the root of *V. amurensis* (named Amurensin H) (Huang, Lin, & Wang, 1999).

There were 3 peaks at 4.868, 4.997, and 5.162 min of retention time with the same quasi-molecular ions of m/z 325 [M-H] and the transition m/z 325→265 generated from the loss of 60 Da from sugar moiety, however, no more fragmentations showed in the negative mode. The positive mode presented a series of fragments formed multiple product ions at m/z 284, 257, 220, 147, and 120. The ion at m/z 284 is generated from the loss of a C₂H₂O group (42 Da) of compounds. 120 Da and 147 Da are the 2 fragments generated from the cleavage at both sides of carbonyl group (Figure 4), respectively. The fragmentation and chromatographic patterns at 4.868 and 5.162 min are the same as 6-*O*-(*p*-coumaroyl)-D-glucopyranoside that we ever isolated with inhibition on α -glucosidase from grape pomace (Sun et al., 2016). The compound at 4.997 min was supposed to be another one *p*-coumaroyl-6-*O*-hexoside.

Peak 5 presented 2 pseudomolecular ions [M+H]⁺ at m/z 453 and [M+Na]⁺ at m/z 475, releasing 2 MS² fragment at m/z 254 [M-162-36]⁺ and 246 [M-162-44]⁺ (Figure 5), corresponding to (epi)catechin monomer with a loss of a hexosyl moiety and 2 water molecular or ethanol fragment, respectively. In negative mode, this compound presented 3 pseudomolecular ions [M+Cl]⁻ at m/z 487 and 489, [M+COOH]⁻ at m/z 497. The compound showed characteristic MS² fragments of (epi)catechin (Galaverna, Sampaio, Barata, Eberlin, & Fidelis, 2015; Kang, Price, Ashton, Tapsell, & Johnson, 2016), so it was tentatively identified as (+)-(epi)catechin-*O*-hexoside.

In positive mode, peak 6 that there are 1 group of pseudomolecular ions M⁺, [M+2H]⁺, and [M+Na+2H]⁺ at m/z 677, 679 and 701; in negative mode, [M+Cl]⁻ and [M+COOH]⁻ at m/z 713 and 715 and 723, was designated as to 10-carboxyl-pyranopeonidin 3-*O*-(6''-*O*-*p*-coumaroyl)-glucoside based on its fragmentation pattern (Figure 6). The ion at m/z 367 loses a coumaroyl-glucoside moiety and more 2 protons that characteristic of proanthocyanidins with doubly-charged ions (Rockenbach et al., 2012). Ion at m/z 340 is corresponding to 10-carboxyl-pyranopeonidin with a loss of carboxyl moiety, at m/z 298 to a peonidin moiety after a loss of pyruvate moiety, at m/z 257 to a peonidin moiety with a loss of carboxyl moiety, and at m/z 220, 176, 166, and 120 to RDA or HFF product ions (Blanco-Vega, López-Bellido, Alía-Robledo, & Hermosín-Gutiérrez, 2011; He et al., 2012). This is 1 adduct product generated from peonidin 3-*O*-(6''-*O*-*p*-coumaroyl)-glucoside and pyruvic acid during wine fermentation (de Freitas & Mateus, 2011; Marquez, Serratos, & Merida, 2013).

During identification, compounds **3** and **5** were not given the exact structures because of insufficient mass spectral information of sugar moiety and no reference compounds as control. Although UHPLC/MS did not overcome the disadvantage of mass spectral in identification of isomers, specially of many glycosides occurring in grape, it confirmed the retention time of active compound in chromatogram, which helpful for further preparation of purified compounds. UHPLC/MS provided a fast and effective analytical method for trace natural compounds.

Conclusion

The chitosan beads immobilized α -glucosidase, was successfully shown activities and able trap inhibitors that could be isolated for elucidation. The technique in combination with UHPLC/MS detection was able to present additional findings of new inhibitors and their proposed structures, viniferifuran (amurensin H), *p*-coumaroyl-6-*O*-hexoside, (epi)catechin-hexoside, 10-carboxyl-pyranopeonidin 3-*O*-(6''-*O*-*p*-coumaroyl)-glucoside, besides *p*-coumaroyl-6-*O*-D-glucopyranoside that ever isolated and identified under bioactivity-guided, from TCEE. Purified elutes from TCEE will be further investigated for separation of desired single compounds based on the study results. Other preparative methods followed by inhibition bioassays may be suggested to elucidate individual relationships and possible mechanism in attaching α -glucosidase enzyme.

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Practical Application

A potential approach enriched and identified α -glucosidase inhibitors of grape pomace. Set-up of UHPLC/MS detection and identification of active compounds provide qualify assessment in developing grape pomace extract into potent dietary supplement and new drug for diabetes.

Figure 1.

(A) Percentage activity of initial α -glucosidase solution compared to the solution after absorption procedure. At 405 nm, the enzyme activity was determined based on total p-nitrophenol release from p-NPG, where the significant lowered activity suggested successful absorption into chitosan beads ($P < 0.05$). Errors were expressed based on SEM. (B) percentage α -glucosidase inhibition activities of TCEE sample before and after absorption, with comparison to eluate and Acarbose control at 50 $\mu\text{g/mL}$. *Eluate showed significant improvement in inhibition level ($P < 0.05$). Errors were expressed based on SEM.

Figure 2.

Total ion chromatogram in positive and negative mode and tentative structures of the major compounds interacted with α -glucosidase from TCEE eluates.

Figure 3. MS/MS spectra of compound 1 with precursor ions m/z 453 $[M+H]^+$ and 475 $[M+Na]^+$ with the proposed fragmentation pathway.

Figure 4.

MS/MS spectra of compounds 2 to 4 with precursor ions m/z 325 [M-H] with the proposed fragmentation pathway at the negative and positive modes.

Figure 5. MS/MS spectra of compounds 5 with precursor ions m/z 453 [$M+H$] and 475 [$M+Na$] with the proposed fragmentation pathway.

Figure 6. MS/MS spectra of compounds 5 with precursor ions m/z 677 [M^+] and 679 [$M+2H$] with the proposed fragmentation pathway.

Table 1

Fragmentation patterns and tentative assignments of compounds interacted with mammal α -glucosidase from Tinta Cão grape pomace extract eluates.

Peak	RT	Formula	MW	Tentative identification	Positive fragmentation (m/z)	Negative fragmentation (m/z)
1	3.933	C ₂₈ H ₂₀ O ₆	452	viniferifuran (amurensin H)	453 [M+H] ⁺ , 475 [M+Na] ⁺ , 367, 359, 305, 249, 227 (100%)	271, 253, 251
2	4.868	C ₁₅ H ₁₈ O ₈	326	<i>p</i> -coumaroyl-6- <i>O</i> -D-glucopyranoside	284, 257, 220, 120	325 [M-H] ⁻ , 265, 113
3	4.997	C ₁₅ H ₁₈ O ₈	326	<i>p</i> -coumaroyl-6- <i>O</i> -hexoside	257, 220, 120	325 [M-H] ⁻ , 265, 113
4	5.162	C ₁₅ H ₁₈ O ₈	326	<i>p</i> -coumaroyl-6- <i>O</i> -D-glucopyranoside	284, 257, 220, 120	325 [M-H] ⁻ , 265, 113
5	5.902	C ₂₈ H ₂₀ O ₆	453	(epi)catechin glucoside	453 [M+H] ⁺ , 475 [M+Na] ⁺ , 254, 246, 159, 156	487/489 [M+Cl] ⁻ , 497 [M+COOH] ⁻ ,
6	6.562	C ₃₄ H ₂₉ O ₁₅ ⁺	677	10-carboxyl-pyranopeonidin 3- <i>O</i> -(6''- <i>O</i> - <i>p</i> -coumaroyl)-glucoside	677 [M] ⁺ , 679[M+2H] ²⁺ , 701[M+Na+2H] ⁺ , 367, 359, 340, 298, 257, 220, 176, 156, 158, 120	713/715[M+Cl] ⁻ , 723 [M+COOH] ⁻ , 113