Bioactivity-based antioxidative components screening and evaluation in grape seed proanthocyanidin extract

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Abstract Grape seed proanthocyanidin extract (GSPE), a type of functional food, possesses potent antioxidant activity. In this study, GSPE protected human embryonic kidney 293 (HEK 293) cells from H₂O₂-induced cell injury and oxidative stress in a dose-dependent manner. The key effective constituents that exerted the most potent antioxidative activity in GSPE were screened by using a modified ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q/TOF MS) integrated 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical cation antioxidative activity analysis system. Two compounds, which were presumed to be Procyanidin B2 and Procyanidin C2, showed obvious antioxidant activity. H₂O₂ scavenging effect of Procyanidin B2 in HEK 293 cells was visualized in situ by a molecular imaging technique via a novel *N*-borylbenzyloxycarbonyl-3,7-dihydroxyphenoxazine (NBCD) fluorescent probe to detect levels of H₂O₂. In conclusion, the application of UPLC-Q/TOF MS integrated modified ABTS radical cation antioxidative activity analysis system and NBCD fluorescent

probe successfully screened out and confirmed the antioxidative components from GSPE.

Keywords Antioxidants · Fluorescent probe · G-quadruplex · Grape seed proanthocyanidins · Oxidative stress

Introduction

Grape seed proanthocyanidin extract (GSPE), a complex of various polyphenols extracted from grape seed, possesses potent antioxidative activity (Bagchi et al. 2014). It has been approved to be sold as a dietary supplement in USA. As grape seed is a by-product or even waste in the food industry such as wine production, the utilization of grape seed extract will benefit both humans and the environment. For example, GSPE was proved to exert preventative effect in 4-nitroquinoline 1-oxide induced oral tumorigenesis in mice. This effect was reported to be associated with its capacity of inhibiting proliferation and inducing apoptosis (Shrotriya et al. 2015).

GSPE possesses potent antioxidative activity. Impei characterized 37 autochthonous *Vitis* accessions through determination of their metabolic profiles, and reported notable antioxidant effects from these samples (Impei et al. 2015). Recently, Gismondi reported free radical scavenging properties of extracts from eight Italian *Vitis vinifera* L. cultivars (Gismondi et al. 2017). Also, there have been many studies performed to test the protective effects of GSPE relative to its antioxidative activity (Fernández-Iglesias et al. 2014; Bao et al. 2015). As H₂O₂ is one of the most stable and abundant reactive oxygen species (ROS) and is considered as a key mediator of cellular and tissue damage induced by oxidative stress, H₂O₂-based methods

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of diagnostics and therapies for different oxidative stress-associated diseases have become an attractive research topic (Lee et al. 2015; Kang et al. 2016). Therefore, it is necessary to establish a rapid and visible method for H_2O_2 detection. However, the effect of GSPE on H_2O_2 has never been intuitively demonstrated. Recently, Han et al. have synthesized a chemicobiological fluorescent probe, *N*-borylbenzyloxycarbonyl-3,7-dihydroxyphenoxazine (NBCD), to detect H_2O_2 levels. This probe is stable under cell culture conditions and can be used for H_2O_2 detection in living cells with a detection limit of 2 μM (Han et al. 2016). The application of this probe in the evaluation of antioxidants will be helpful to researchers.

Although so many pharmacological effects of GSPE relative to its antioxidative capacity have been reported (Fernández-Iglesias et al. 2014; Bao et al. 2015), the most potent components in it remain to be identified. Therefore, a sensitive and stable screening system is needed to solve this problem. In the preparation of 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical cation, which was widely used to detect antioxidants (Suttirak and Manurakchinakorn 2014), poor stability hampered the application of it in a high-throughput screening. In a recent report from Kong's laboratory (Jia et al. 2012), a G-quadruplex DNzyme-catalyzed method was applied in the preparation of ABTS radical cation. This modification made possible for the large-scale preparation of $\text{ABTS}^{\cdot+}$. Thus, it could be applied in the bioactivity-integrated ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q/TOF MS) analysis system established by our laboratory for antioxidants screening.

In this study, we demonstrated the potent antioxidative abilities of GSPE in H_2O_2 -induced human embryonic kidney 293 (HEK 293) cell injury. Then, we identified the most potent components with antioxidative activity in GSPE by using the modified UPLC-Q/TOF MS integrated $\text{ABTS}^{\cdot+}$ antioxidant screening analysis system. Finally, we confirmed the screening results by an in situ imaging of H_2O_2 levels using the NBCD fluorescent probe.

Materials and methods

Chemicals and reagents

GSPE was kindly provided by Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China). It contains approximately 56% dimeric proanthocyanidins, 12% trimeric proanthocyanidins, 6.6% tetrameric proanthocyanidins, small amounts of monomeric and high molecular oligomeric proanthocyanidins, and flavanols. Malondialdehyde (MDA), lactate dehydrogenase (LDH), and

catalase (CAT) assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and H_2O_2 were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin and phosphate buffer saline (PBS) were purchased from Gibco BRL Life Technologies (Rockville, MD, USA). LC grade acetonitrile was purchased from Fisher (Pittsburgh, PA, USA); LC grade formic acid was purchased from Acros Organics (Geel, Antwerp, Belgium); LC water was purified by a MilliQ system (Millipore Corp, Bedford, MA, USA). The fluorescent probe NBCD was kindly provided by Professor Zheng Yin, Nankai University (Tianjin, China) (Han et al. 2016). $\text{ABTS}^{\cdot+}$ reagent was kindly provided by Professor Deming Kong, Nankai University (Tianjin, China) (Jia et al. 2012). Sodium ascorbate was purchased from Macklin (Shanghai, China). Trolox, a water-soluble form of vitamin E, was purchased from Shengyang Ranger (Beijing, China). The purity of all the other reagents used was analytical grade.

Cell culture

HEK 293 cells, purchased from American Type Culture Collection (Rockville, MD, USA), were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin G and 100 $\mu\text{g}/\text{mL}$ streptomycin) in a humidified atmosphere containing 5% CO_2 at 37 °C. For all experiments, cells were grown to confluence in 96-well plates and starved for 24 h in DMEM supplemented with 1% FBS before treatments.

Determination of cell viability and oxidative stress

According to our preliminary experiments, 400 μM of H_2O_2 was used in this in vitro oxidative stress model. Cells were pretreated with different concentrations of GSPE (800, 200 or 50 $\mu\text{g}/\text{mL}$). After 1 h, the cell culture medium was displaced with 400 μM of H_2O_2 dissolved in fresh DMEM, and cells were incubated for 5 h. The MTT assay was performed to evaluate cell viability in different groups. No significant toxicity was observed at any of the concentrations of GSPE used in the in vitro experiments.

The supernatants of the cells were collected for assays of LDH, MDA, and CAT using their respective kits based on colorimetry. LDH activity was determined based on the catalysis of lactic acid to form pyruvic acid, which reacted with 2,4-dinitrophenylhydrazine to form brownish red pyruvate dinitrophenylhydrazone. The activity of CAT was calculated based on the reaction of H_2O_2 with ammonium molybdate that produced a light yellow compound. The concentration of MDA was calculated based on the formed

red product using thiobarbituric acid (TBA) method. The assays were performed according to the methods described by the manufacturer.

UPLC analysis and sample preparation for activity assay

A Waters Acquity UPLC System (Waters Co., USA) equipped with a photodiode array detector was used. The detection wavelength was in the range of 190–400 nm. The system was controlled using MassLynx V4.1 software (Waters Co., USA). An Acquity BEH C₁₈ column (2.1 × 100 mm, 1.7 μm) was used for the separation. The mobile phase was a gradient elution system of A (0.1% formic acid) and B (CH₃CN), and the elution was programmed as follows: 2–8% B at 0–10 min, 8–18% B at 10–20 min, 18–45% B at 20–25 min, 45–70% B at 25–30 min, and 70–2% B at 30–32 min. The flow rate was 0.40 mL/min, and the column temperature was maintained at 30 °C.

One μL of GSPE solution (200 μg/mL) was injected into the UPLC system. A part of the UPLC fractions was used for Q-TOF-MS analysis, and the others were collected into a 96-deep-well plate every 0.5 min and evaporated to dryness in a vacuum drying oven at 35 °C. The residues were dissolved in ddH₂O (50 μL each well). The separation process was repeated three times for analysis in the following antioxidant screening analyses.

Q-TOF-MS analysis

Accurate mass measurements and MS/MS were performed on a Waters Q-TOF Premier with an electrospray ionization (ESI) system (Waters MS Technologies, Manchester, UK) operating in the negative mode. The electrospray capillary voltage was set to 2.5 kV. The sample cone voltage was set to 30 V. High-purity nitrogen was used as the nebulization and auxiliary gas. The nebulization gas was set to 600 L/h at 350 °C, the cone gas set to 50 L/h, and the source temperature was 110 °C. The Q-TOF premier acquisition rate was 0.1 s, with a 0.02 s inter-scan delay. The instrument was operated with the first resolving quadrupole in a wide pass mode (50–1000 Da). Leucine-enkephalinamide acetate (200 μg/L) was used as the lock mass ($[M - H]^- = 553.2775$).

Antioxidant screening analysis using ABTS^{•+} system

Scavenging activity of the ABTS^{•+} radical is often used to detect the existence of antioxidants. In our study, determination of the antioxidant activity was performed as a previously reported system (Jia et al. 2012). In this system, the G-quadruplex-forming oligonucleotide strand CatG4

was used for the preparation of ABTS^{•+} in replacement of natural peroxidases that were used in traditional methods. ATP was also added to the system to stabilize the pre-formed ABTS^{•+}. In this study, 50 μL of pre-formed ABTS^{•+} solution was mixed with 10 μL of individual UPLC eluate fractions or total GSPE solution (200 μg/mL), and then diluted to 100 μL with 10 mM Tris-HCl buffer (pH 7.0). The blank control was the same reaction system except that samples were substituted with ddH₂O. Similarly, sodium ascorbate solution (200 μg/mL) was used as the positive control. The mixtures were briefly vortexed, and the absorbance at 734 nm was measured 5 min later. The antioxidant activity was expressed as the decrease of absorbance: absorbance = $A_{\text{blank}} - A_{\text{sample}}$.

H₂O₂ detection using a NBCD fluorescent probe

A novel fluorescent probe (NBCD) was used to detect H₂O₂ levels in cells due to the release of resorufin that displayed red fluorescence from NBCD after a series of reactions with H₂O₂. A Leica TDS SP8 laser scanning confocal microscope was used to detect fluorescence intensity according to the methods described by Han et al. (2016) with some modifications. Briefly, HEK 293 cells were incubated with 10 μM NBCD for 15 min at 37 °C, followed by washing three times with PBS. Cells were then incubated with 400 μM H₂O₂ for 1 h with or without GSPE (200 μg/mL), Procyanidin B2 (10 μM), or Trolox (10 μM). Trolox was used as the positive control. Fluorescence intensity change was also determined in a cell-free system as described by Han et al. (2016) in supporting information.

Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM) for the number of observations. One-way analysis of variance (ANOVA), followed by the Dunnett post hoc test using the SPSS 11.5 program (SPSS Inc., Chicago, IL, USA), was used for comparison among the groups. A *P* value of <0.05 was considered statistically significant.

Results and discussion

In this study, the effects of different concentrations of GSPE pretreatment on cell viability after H₂O₂ injury were tested using the MTT assay. As shown in Fig. 1a, H₂O₂ decreased the cell viability to approximately 45%, taking the optical density of untreated control cells to be 100%. GSPE could prevent cell death and enhance the cell viability in a dose-dependent manner, indicating potent

Fig. 1 GSPE pretreatment ameliorated oxidative stress in H_2O_2 -injured HEK 293 cells. **a** Effects of different concentrations of GSPE pretreatment (800, 200 or 50 $\mu\text{g}/\text{mL}$) on cell viability after H_2O_2 injury tested by the MTT assay. **b–d** Effects of different concentrations of GSPE pretreatment on LDH release (**b**), MDA (**c**), and CAT (**d**) in cell culture supernatants after H_2O_2 injury. Each bar represents mean $\pm$ SEM. $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$ versus H_2O_2 , $^{##}P < 0.01$ versus H_2O_2 ($n = 6$)

protective effects against H_2O_2 -induced injury. The release of LDH was used as another parameter to evaluate cell viability, reflecting the integrity of the cell membrane. The results showed that LDH release in the H_2O_2 -treated group (298.69 ± 20.05 U/L) was much more than the LDH release in the control group (41.88 ± 6.45 U/L). In contrast, GSPE pretreatment (800, 200 or 50 $\mu\text{g}/\text{mL}$) significantly reversed the effect and lowered LDH release to 49.77 ± 9.08 , 85.77 ± 4.70 and 138.24 ± 15.49 , respectively (Fig. 1b). MDA level in cell culture supernatants was used as an index of oxidative stress. CAT is one of the most important endogenous antioxidative enzymes to scavenge reactive oxygen species in cells. The activity of CAT reflects the antioxidative ability of the cells. As shown in Fig. 1c, the MDA level was significantly increased (1.49 ± 0.10 nmol/mL) when compared with the control group (0.21 ± 0.03 nmol/mL). GSPE (800, 200 or 50 $\mu\text{g}/\text{mL}$) dose-dependently decreased MDA level (0.15 ± 0.01 , 0.43 ± 0.02 and 0.69 ± 0.08 , respectively). CAT activity was markedly lowered by H_2O_2 treatment (0.63 ± 0.30 U/L versus 3.25 ± 0.86 U/L, $P < 0.01$), while GSPE pretreatment reversed this effect dose-dependently (Fig. 1d). These results suggested that GSPE showed a strong antioxidative ability in this in vitro model, and led us to the following bioactivity-based screening of antioxidative components.

Among the various assays used to analyze the antioxidative effects of phenols, the most common one is the ABTS⁺ analysis system. The oxidant ABTS⁺ is superior to others in its high solubility in water, which is the common solvent used in these antioxidant assays. Furthermore, it also has a standard reduction potential which is high enough to oxidize phenols (Walpen et al. 2016). These

characters make it suitable for the evaluation of antioxidants in natural products (Suttirak and Manurakchinakorn 2014; Zhen et al. 2016). To be noticed, we chose a novel ABTS⁺ antioxidative activity analysis system, G-quadruplex DNAzyme-catalyzed ABTS- H_2O_2 system, which was first reported by Kong's laboratory (Jia et al. 2012). In the original analysis methods, protein enzymes such as metmyoglobin and horseradish peroxidase were used to evaluate antioxidative activities by investigating the effects of antioxidants on them. While in this novel system, G-quadruplex DNAzyme (also known as peroxidase-like G-quadruplex-hemin DNAzyme) was applied. This highly catalytic complex was formed by adding hemin to CatG4, a G-rich oligonucleotide with a G-quadruplex structure. Adenosine triphosphate (ATP) was also used to decrease the destruction of the CatG4/hemin complex. This system can be prepared on a large scale because it is more stable, and the lifetime of ABTS⁺ is significantly prolonged. The critical advantages of this system over the original systems have been thoroughly demonstrated in their paper (Jia et al. 2012). The application of this method greatly facilitates the establishment of a high-throughput screening system, and the results are easily judged.

By using our UPLC-Q/TOF MS integrated modified ABTS⁺ antioxidative activity analysis system, GSPE was used for further screening and identification. Figure 2a, b were UPLC-UV chromatograms of GSPE and the total ion current chromatograms in the negative ESI mode, respectively. The bioactivity fingerprint for GSPE was shown in Fig. 2c. The two compounds that showed obvious antioxidant activity were proposed to be Procyanidin B2 and Procyanidin C2, a proanthocyanidin dimer and a proanthocyanidin trimer respectively. The identification of these

Fig. 2 The UPLC/Q-TOF-MS and antioxidant activity analysis of GSPE. **a** UPLC-UV chromatograms of GSPE TIC data, **b** total ion current chromatograms in the negative ESI mode, **c** bioactivity chromatograms obtained via the ABTS system for antioxidant activity

analysis. The peak numbers are consistent with the peaks in the chemical structures. **d** Chemical structures of the bioactive compounds screened out in GSPE

Table 1 MS/MS data of (–) ESI-MS and the identification of the bioactive compounds of GSPE

Peak no.	t_R (min)	Identification	M/Z ($[M-H]^-$)	MS^2	m/z	Composition
1	8.86	Procyanidin B2	577.1405	1155 $[2 M-H]^-$, 577 $[M-H]^-$, 425 $[M-C_8H_8O_3-H]^-$	578.1424	$C_{30}H_{26}O_{12}$
2	12.85	Procyanidin C2	865.1980	865 $[M-H]^-$, 577 $[M-catechin]^-$, 407 $[M-catechin-C_8H_8O_4-H]^-$	866.2058	$C_{45}H_{38}O_{18}$

compounds was based on the exact molecular weight and published literature data. The chemical structures of the bioactive compounds are shown in Fig. 2d, and the MS/MS data were listed in Table 1.

Diverse bioactivity-integrated UPLC-Q/TOF MS analysis systems have been established in our laboratory. An analysis system coupled with the NF- κ B luciferase reporter assay system has been applied successfully to the identification of NF- κ B inhibitors in many types of plant extracts and multiherb medicines (Jiang et al. 2014; Gao et al. 2015; Han et al. 2015). Recently, our UPLC-Q/TOF MS system combined with the dual-luciferase reporter assay for intracellular calcium also successfully screened out

synergistic bronchodilators from a traditional Chinese medicine (Hou et al. 2014). The previous experience guaranteed the successful establishment of this UPLC-Q/TOF MS integrated ABTS⁺⁺ analysis system for antioxidants. In fact, an HPLC-or UPLC-coupled antioxidant analysis system has been used in recently published articles. For example, Lee et al. (2014) reported the successful trial of an online screening HPLC-ABTS assay in the rapid characterization of bioactive compounds in plant extracts. In a recent work of Peng et al. (2016), an online UPLC-Q/TOF MS-ABTS⁺⁺ system was used to evaluate bioactive compounds with antioxidative ability in the Danshen injection. The application of online analysis technique in

our UPLC-Q/TOF MS integrated ABTS⁺ analysis system might further benefit the efficiency of antioxidant screening.

As is known, GSPE contains proanthocyanidins which are composed of dimers or trimers of (+)-catechin and (–)-epicatechin (Shi et al. 2003). Proanthocyanidins have been demonstrated to exert potent antioxidative activities. For example, the oligomeric proanthocyanidin complexes (OPCs) extracted from French maritime pine bark are widely used as a type of functional food with therapeutic effects, such as an OPCs product with the trademark of Pycnogenol® (Ezzikouri et al. 2015; Jankyova et al. 2016). Similarly, GSPE, a mixture of OPCs extracted from grape seed, might exert similar effects. Our screening results were in good accordance with previous reports that proanthocyanidins were active components (Huang and Lai 2016), and clearly demonstrated that Procyanidin B2 and Procyanidin C2 were the two components with obvious antioxidative activity.

Subsequently, Procyanidin B2 (PCB2) was chosen as the representative compound to confirm the aforementioned screening results of antioxidative capacity. As shown in Fig. 3, basal H₂O₂ could be detected in untreated HEK 293 cells after NBCD incubation, while H₂O₂ stimulation significantly increased the fluorescence intensity in the HEK 293 cells. PCB2 decreased the fluorescence dramatically, and the effect was comparable to the effect observed in Trolox, the positive control. In a cell-free reaction system, PCB2 also scavenged H₂O₂ chemically in a dose-dependent manner (Fig. 4). These results demonstrated the potent oxidative stress scavenging effects of PCB2 and confirmed the antioxidant screening results. NBCD probe is a helpful tool in detection of H₂O₂. The

Fig. 4 The fluorescence intensity normalized to the control tested in a cell-free reaction system. Treatments included 400 μ M H₂O₂, Trolox (10 μ M), PCB2_H (high dose of PCB2, 100 μ M) and PCB2_L (low dose of PCB2, 10 μ M). Each bar represents mean $\pm$ SEM. ** P < 0.01 versus control; ## P < 0.01 versus H₂O₂ (n = 3)

application of the fluorescent probe NBCD in this experiment made the result visible and easily judged. At present, we have successfully applied it in H₂O₂ detection in fruit flies (data not shown) and hope to expand its application to more in vivo experiments.

It is concluded that PCB2 exerts potent H₂O₂ scavenging activity through redox reactions. As the major reduction site, ortho phenolic hydroxyls in B rings of PCB2 are presumed to serve as H donors to capture the free radicals generated in the oxidation process. Then, the free radicals formed in PCB2 can remain stable through the formation of intramolecular hydrogen bonds, semiquinone free radicals, or even *o*-quinone. Thus, the chain reaction of free radicals is interrupted (Sato et al. 2010). The mechanism underlying

Fig. 3 Representative confocal images of HEK 293 cells after the NBCD probe incubation, followed by washing and different treatments. Cells were stimulated with or without 400 μ M H₂O₂, GSPE (200 μ g/mL), PCB2 (10 μ M) or Trolox (10 μ M). Scale bars 10 μ m

Fig. 5 The predicted mechanism underlying the application of NBCD probe in demonstrating the antioxidative capacity of PCB2 via detection of fluorescence change of cells

the process of the fluorescence changes of cells with different treatments after the NBCD probe incubation is shown in Fig. 5. The therapeutic effects of PCB2 have been demonstrated by others. For example, Chang et al. (2007) reported that PCB2 showed protection in cardiomyocytes exposed to ischemia/reperfusion. Procyanidin B2 3,3''-di-*O*-gallate (B2G2) is identified as most active constituent of GSPE in the protection effect against prostate cancer. The synthesized B2G2 exerted equivalent biological efficacy against human prostate cancer cell lines as B2G2 isolated from GSPE (Tyagi et al. 2014). Thus, there are possibilities that PCB2 might be used as a complementary therapy to treat oxidative stress-associated diseases, such as ischemia/reperfusion injuries, in clinics. As to Procyanidin C2, the other compound that was screened out by our analysis system, this is the first report stating its antioxidative ability to the best of our knowledge. Further studies are needed to explore its usage as an antioxidant.

This is only a first try of the rapid screening and confirmation of antioxidants from a type of functional food, namely GSPE. The applicability of this system in the antioxidants screening from other functional food still needs to be confirmed. Furthermore, the integration of UPLC-Q/TOF with other antioxidant assay methods, such as DPPH radical scavenging assay (Kumar et al. 2016), might also be applied to the analysis of functional food and

natural products in the future. This method will make the bioactivity-based antioxidative components screening system more comprehensive and reliable.

Conclusion

Our results indicate that GSPE attenuated oxidative stress-induced cell injury at least partially due to its potent antioxidant activity. Based on its bioactivity, a UPLC-Q/TOF integrated modified ABTS^{•+} screening system and an H_2O_2 -sensitive NBCD probe were applied to the screening of its key components. By using these methods, Procyanidin B2 and Procyanidin C2 were screened out as the most effective components in GSPE. As a type of functional food widely used, GSPE and its antioxidative components will probably be developed to be a vital complementary therapy in the treatment of oxidative stress-associated diseases in the clinic. The bioactivity-based antioxidative components screening system might also be used in the research of other natural products.

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