In vitro study of antigrowth capacity and antiacid capacity on *Sreptococcus sobrinus 6715* of sorghum procyanidin dimmers

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Abstract: The bacteria grow in oral cavity and product acids, which could induce dental caries. In this study, in order to obtain the relationship between procyanidin dimers from sorghum episperm (sorghum procyanidins, SPC) and its anticaries effect. The extract of SPC purified by macroporous resin was divided into three parts by gel chromatography, marked as GPC-1, GPC-2, and GPC-3 in order. The ESI-MS and MS/MS analysis indicated that the main composition of GPC-2 was procyanidin dimers. In addition, the capacities of antigrowth and antiacid on *Sreptococcus sobrinus 6715* were analysised to investigate the effect of SPC dimers in protecting against dental caries. The results indicated that the minimum inhibitory concentration (MIC) of SPC dimers was 16 mg/mL. Furthermore, the SPC dimers had notable preventive effect ($P<0.05$) against the acid production of *Sreptococcus sobrinus 6715* compared with the control group, which suppressed in a dose-dependent manner by pH decline. These findings indicated that SPC dimers had potential to be used as anticaries preventive medicine due to its strong capacity of antigrowth and antiacid.

Keywords: Procyanidin dimers, multi-stage MS, antibacterial activity, caries.

INTRODUCTION

Dental caries is one of the most prevalent epidemic and chronic mammalian disease all over the world. For dental caries, varieties of preventable and treatable measures have been explored, among which drug treatment is an effective and frequently-used method (Marthaler *et al*., 2005; Twetman *et al*., 2003). Polyphenols are one of the most common groups of substances produced in plants and it has long been known that they have a broad range of health promoting activities (Tapiero *et al*., 2002). Polyphenols can combine with proteins, and microbial growth needs certain enzyme and protein, as a result, the microbial metabolism may be inhibited by polyphenols (Xiao *et al*., 2013). Many studies have indicated that plant polyphenols showed significant inhibitry effect against dental caries, such as antigrowth, anti-acid, and antiadhesive capacities (Otake *et al*., 1991; Schmid *et al*., 1988; Matsumoto *et al*., 1999). Procyanidins, a kind of plant polyphenols, may serve as new antimicrobials with a broader antibiogram, better inhibition of enzyme activity and stronger proteins binding capacity (Tao, 2009). Thus, it is pertinent to focus on procyanidins for their role in dental caries prevention.

Dental caries, a chronic infectious disease, is mainly induced by *Sreptococcus sobrinus* and *Sreptococcus mutans* (Hattori *et al*., 1990 and Gibbons 1989). The bacteria inhabit the surface of teeth and produce acids causing demineralization of teeth, which cause caries eventually (Scheie *et al*., 1996). Thus, control of the bacterial growth and acid production are essential to prevent caries. Otake *et al* (1991) confirmed that a crude green tea extract could significantly induce the adhesion of *S. mutans* to saliva-coated hydroxyapatite. A recent study demonstrated Cranberry juice could reduce hydrophobicity of cell surface of the *S. mutans* and *S. sobrinus*' cells, interfering with adhesion (Yamanaka *et al*., 2004). The ethanol extract of *Aralia continentalis* was also reported for its inhibitory activity on the growth and acid production by *S. mutans* (Lee *et al*., 2011). Sorghums (*Sorghum bicolor Moench*) are rich in phenolic compounds, which include phenolic acids, flavonoids, and condensed tannins (Dykes *et al*., 2006). Schmid *et al*. (1988) found that sorghum showed more notable capacity on caries' prevention. *Sorghum procyanidins* could significantly inhibit acid production by *S. sobrinus (Ingbritt C)* and *S. mutans 6715*, besides the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were 8 and 16 mg/ml, respectively (Liman *et al*., 2011). The complicated structure and the difficulty of isolation and identification of procyanidins limited the further research of the mechanism for the antibacterial and anticaries activities. Most of the above studies involved crude extract or a mixture of different degree of procyanidins as materials to evaluate these activities. Yet the physiological activity of procyanidins is closely related to procyanidins' molecular size and structure, and many studies have shown that the *Oligomeric proanthocyanidins* had higher activity (Lotito *et al*., 2000; Ariga *et al*., 1990). Mass spectrometry played an increasingly important role in the identification of natural active component. At the early stage in mass spectrometry technology, electron impact mass spectra (EI-MS) and fast atom bombardment mass spectrometry (FAB-MS) were the main methods. With the advent of soft ionization technique, electrospray ionization mass

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spectrometry (ESI-MS) are widely used in the analysis of natural products research, especially in the case of thermolabile compounds (Mauri *et al*., 1999). Its MS mainly produce molecular ion peak, and MS^n can provide structural information of compounds, rapid and effective modern analysis method to study the complex system. Many researchers used the multi-stage MS technology as an effective technical means to analyze the structure of procyanidins (Friedrich *et al*., 2000; Liwei *et al*., 2003; Maldini *et al*., 2011).

The present work was aimed to use ADS-17 macroporous resin and Toyopearl HW-40s gel chromatography to purify and separate the procyanidins, which were extracted from China sorghum episperm, to obtain different molecular weight SPC, especially highly reactive procyanidin dimers. Direct flow injection/electrospray ionization/ion trap tandem mass spectrometry was used to detect its composition. The effects of procyanidin dimers on *S. sobrinus 6715*, including its antigrowth ability and antiacid capacity were investigated.

MATERIALS AND METHODS

Reagents and materials

Reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Tryptone and soy peptone were purchased from Beijing Shuangxuan Microorganism Medium Product Company (Beijing, China). ADS-17 macroporous resin was purchased from Tianjin Synthesis Science and Technology Company (Tianjin, China). Toyopearl HW-40(s) gel was purchased from Tosoh Corporation (Tokyo, Japan). *Sorghum bicolor moench* was collected from the Northeast China.

Isolation and purification of SPC

The procyanidins in sorghum episperm (150g) were extracted thrice by 1600ml ethanol (70: 30, *v/v*) heated in water-bath at 80°C for 90min. After removing the residue, the filtrates were concentrated in vacuum by a rotary evaporator (Temperature was kept below 40°C).

The concentrate (approximately 40 ml) was loaded into an ADS-17 macroporous resin column (30 cm × 5 cm ID). First elution was eluted by distilled water to remove water-soluble impurities, and again eluted with 3BV of ethanol/water mobile phase (40:60, *v/v*) at 3 ml/min. The ethanol/water eluant was collected, and then condensed by vacuum rotary evaporator (maintained the temperature at about 36°C) and lyophilized (Liman *et al*., 2011).

With the concentration of 0.1g/ml, the samples were filtered by 0.45 μm membrane, and then the filtrates (approximately 1ml) were loaded into a Toyopearl HW-40(s) gel column (42 cm × 2 cm ID). Bulk fractions, eluted by methanol, were collected at a flow rate of 0.6 ml/min, for about 12-16h. An absorbance curve at 280 nm and 350 nm of UV detection (Shimadzu Corporation, Japan, UV-1750) vs. fraction number was constructed.

Analysis of mass spectrum, MS^2, and $MS^3$of the GPC-2

After dissolving approximately 5 mg of GPC-2 powder in 5 ml of methanol, the solution filtered by a 0.45 μm filter membrane. The sample was injected into mass spectrometer (Agilent 1100, US) directly. All MS, MS^2 and MS^3 were handed under the following conditions: negative ion mode, ion source type as ESI, masses scanning from 50 to 2200 *m/z*, dry temperature as 350°C, dry gas set as 10L/min, nebulizer set as 40psi and capillary voltage as 3000 V.

Antimicrobial activity

Bacterial strains and growth conditions

S. sobrinus 6715 obtained from Oral Medical Hospital of Hubei Province (Wuhan, China), cryopreserved at -80°C and cultured in tryptic soy broth (TSB). After 48 h recovery in TSB, *S. sobrinus 6715* were incubated anaerobically in TSB for 18h (Ranran *et al*., 2007).

Determination of the MIC of SPC dimers on S. sobrinus 6715

The concentrations of SPC dimers at the range of 32, 16, 8, 4, 2, 1 and 0.5 mg/ml were obtained .by dissolving the SPC dimers powders in sterile TSB medium using double-diluting method. Then SPC dimers-containing medium with different concentrations were mixed with the same volume of bacteria-grown medium, the concentation of SPC dimers-contained solution turned out to be16, 8, 4, 2, 1, 0.5 and 0.25 mg/ml, eventually SPC dimers-contained solutions were incubated an aerobically in tubes at 37°C for 24 h. Each concentration of SPC dimers was tested in triplicate. The mixed medium without SPC dimers was used as the positive control, while the mixed medium containing SPC dimers and fluid medium but without bacteria was used as the negative control.

The influence of SPC dimmers on acid production of S. sobrinus 6715

According to the result of MIC determination on *S. sobrinus 6715*, selected 7 dilution below MIC to determine the effect on acid production. 0.2ml of the bacteria-containing medium (10^6 CFU/ml) was mixed with 2ml of SPC dimers-contained TSB under aseptic circumstances, and determined the initial pH at the same time, and then cultivated anaerobically in tubes at 37°C for 24 h. The final pH of culture fluid was evaluated by φ70 digital acidity meter. Each concentration was meatured in quadruplicate and then the ΔpH ($pH_{initial} - pH_{final}$) was calculated (Ling, 2008).

STATISTICAL ANALYSIS

Student t-test was used to calculate the significance differences between the experimental group and the control group (SPSS 11.5 for Windows, SPSS Inc., Chicago, IL). Statistically significant values were defined as $P<0.05$.

RESULTS

Separation of SPC

Each tube of the elute, which was eluted through the Toyopearl HW-40 (s) gel column first and then collected by the fraction collector, was measured at 280 nm and 350 nm for absorbance, the elution curve shown in fig. 1.

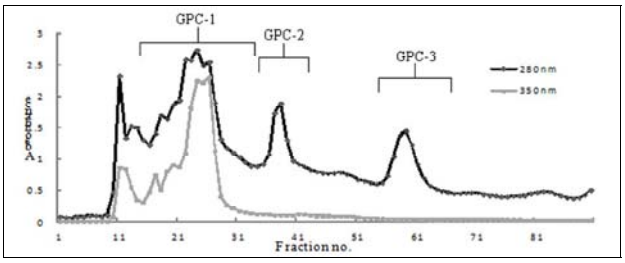

Fig. 1: Elution curve of absorbance Vs tubes/fractions

The purified product was divided into three parts by gel chromatography, marked as GPC-1, GPC-2, and GPC-3 in order (fig. 1). Based on the UV spectrum characteristics of structure of proanidinscy, as a kind of flavones (fig. 2), GPC-1 could be inferred as flavone, GPC-2 and GPC-3 were procyanidins.

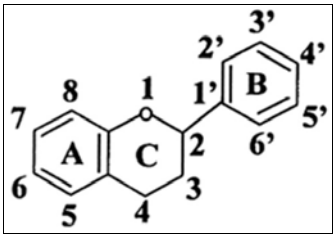

Fig. 2: Nuclear structure of flavones

ESI-MS, ESI-MS^2, and ESI-MS^3 analysis of GPC-2

Fig. 3A shows the ESI-MS fingerprint in the negative ion mode. The main molecular ion peak in the MS spectrum is *m/z* 577.21, it may be procyanidin dimers. For proving the inference described above, second-order ESI-MS/MS experiments for confirming GPC-2 were performed. The MS^2 spectrum of the ion at *m/z* 577.21 (fig. 3B) showed the main product ions at *m/z* 558.95, 451.01, 425.01, 406.96 and 288.88, and then chose the most typical fragment ion at *m/z* 425.01, which exhibited the highest abundance, to analyze by MS^3. Fig. 3C shows the ESI-MS^3 fingerprint of fragment ion at *m/z* 425.01, and the major product ions at *m/z* 406.90, 380.96, and 272.86.

The MIC of SPC dimers on S. sobrinus 6715

The inhibitory effects of SPC dimers on MIC are concentration-dependent, and SPC dimers showed their minimum activity at the concentration of 16 mg/ml. It turned out that SPC dimers had an inhibition effect on the growth of *S. sobrinus 6715*.

The affection of SPC dimers on acid production of S. sobrinus 6715

The results of the inhibition effect of SPC dimers on acid production of *S. sobrinus 6715* are shown in table 1.Accordingly ,as compared to the control, in the selected concentration range (0.25~16 mg/ml), as the concentration increases, **Δ**pH decreases gradually, and SPC dimers significantly inhibited *S. sobrinus 6715* on acid production ($P<0.05$).

DISCUSSION

Ultraviolet absorption spectroscopy has long been an effective means to indentify the chemical structure. Plant polyphenols have a strong absorption in the UV area because their structure contains benzene ring .The UV spectrum of most flavone compounds have two major absorption peaks, the one in 240 ~ 280 nm range, caused by benzoyl system of ring A, known as band II; the other in 300 ~ 400 nm known as the band I, caused by cinnamate system of ring B (fig. 2). The main difference between the UV spectrum of some flavanols kinds of polyphenols, such as procyanidins, and the fourth carbonyl flavone is the absorption of benzene ring with band B in ring A and B. Thus, in addition to the absorbance of the band E near benzene ring at 200 nm, it is often only in a single peak form in ultraviolet. The λ_{max} of typical flavanols substances is usually in the vicinity of 280nm in methanol solution, respectively (Bi *et al*., 2000). The GPC-2 separated by Toyopearl HW-40(s) gel permeation chromatography was confirmed as procyanidins initially.

Mass spectrometry is an important approach to identify substances, such as EI-MS, FAB-MS, MALDI-TOF-MS, ESI-MS. A normal-phase HPLC method coupled with on-line MS was used to separate and identify monomeric and *Oligomeric proyanidins* present in cocoa and chocolate (Hammerstone *et al*., 1999). The wine tannin oligomers were analyzed by liquid chromatography coupled to EI-MS (Fulcrand *et al*., 1999). Dimers, trimers, tetramers, pentamers and hexamers of procyanidins and catechin/epicatechin monomers were purified by macroporous resin and identified by ESI-MS/MS (Liman *et al*., 2011). In order to obtain a rapid qualitative characterization of GPC-2, separated by Toyopearl HW-40(s) gel permeation chromatography. ESI-MS analysis was performed in advance, MS^2 and MS^3 experiments were also performed for confirming the fragmentation pathway of the main molecular ion peak in the MS spectrum.

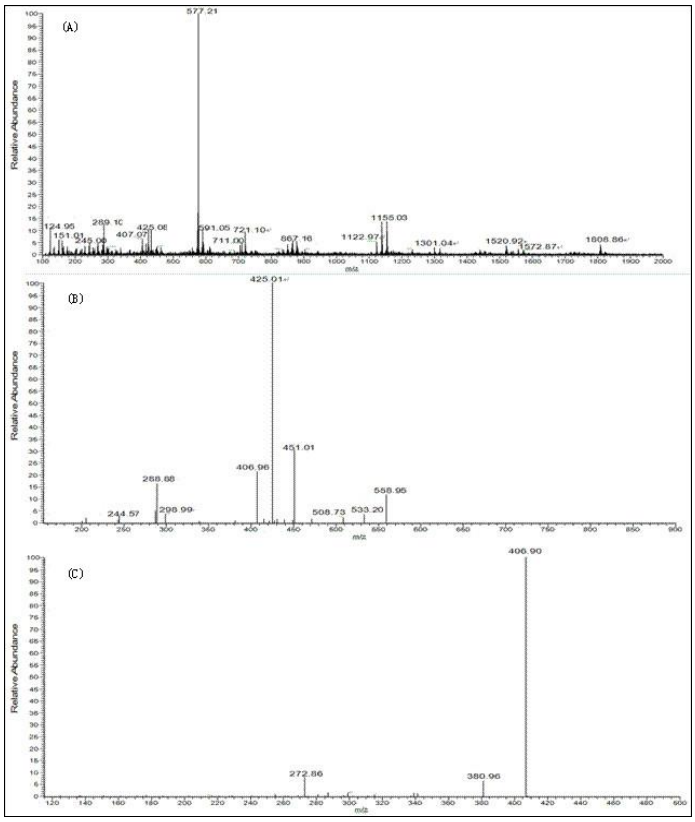

Fig. 3: Negative ionization mass spectrum of GPC-2 (A), ESI-MS^2 (negative ion mode) spectra of *m/z* 577.21 (B), ESI-MS^3 (negative ion mode) spectrum of *m/z* 425.01 (C)

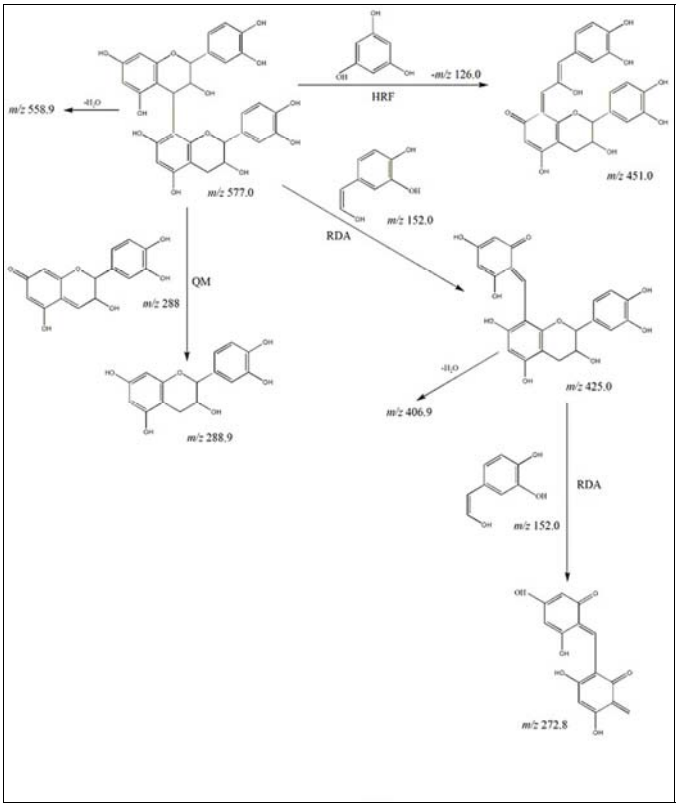

Fig. 4: Fragmentation pathway of a procyanidin dimer detected in sorghum episperm

Table 1: MS^2 and MS^3 analysis of procyanidin dimer fragment ions

	$[M-H]^-$(*m/z*)	Cracking mechanism
MS^2 (*m/z*) 577.08	558.95	Loss of a molecule of H_2O (-18)
	451.01	occurs HRF (-126)
	425.01	occurs RDA (-152)
	406.96	occur RDA and then loss of a molecular of H_2O (-152-18)
	288.88	occurs QM (-288)
MS^3 (m/z) 425.01	406.90	Loss of a molecule of H_2O (-18)

Table 2: Inhibitory effect of SPC dimers on acid production by *S. sobrinus* 6715

SPC dimers concentration (mg/mL)		ΔpH
Sample	16	0.33±0.03*
	8	0.35±0.01*
	4	0.42±0.03*
	2	0.42±0.07*
	1	0.79±0.02*
	0.5	1.08±0.18*
	0.25	1.67±0.08*
Control group	0	1.79±0.03

* Represent $P<0.05$ when compared with the control group, n=3

The fragmentation pathway of the dimer is shown in fig. 4. A procyanidin dimer based on an extension unit and a terminal unit. The heterocyclic ring of the flavan-3-ol units fragment through retro-Diels-Alder (RDA) or heterocyclic ring fission (HRF) mechanism or through Quinone Methide (QM) fragmentation of the interflavonoid bond (Krueger *et al*., 2003). The RDA fragmentation of the dimer produced *m/z* 425.01, and the *m/z* of 409.96 results from water removal of *m/z* 425.01. The HRF of the dimer produced *m/z* 451.01. The QM leads to the production of *m/z* 288.88 (Jingsong *et al*., 2009). After analysis of the main molecular ion ($[M-H]^-$ of *m/z* 577.08) of the MS spectrum through MS^2 and MS^3, the results showed (table 2) that each fragment ion from molecular ions was identical with the associated procyanidin dimers. Therefore, it is determined that GPC-2 consists mainly of procyanidin dimers.

According to Liman's results (Liman *et al*., 2011), SPC showed better antimicrobial activities with a MIC of 8 mg/ml. However the result of SPC dimers is between the MIC of 90% tea polyphenols and carboxymethyl chitosan, the former is 2.5 mg/ml (Ranran *et al*., 2007), and the latter is 60 mg/ml (Tao *et al*., 2003). Accordingly, SPC dimmers was an effective antimicrobial.

S. sobrinus 6715 could produce acids while fermenting the carbohydrates, which will induce pH values falling below a critical value, and eventually result in demineralisation of tooth tissues (Scheie *et al*., 1996). Lee *et al*. (2011) examined the effect of the ethanol extract of *Aralia continentalis* on acid production by *Streptococcus mutans*. The inhibitory effect of Procyanidins in buckwheat on acid production was also discussed by Tao (2009). Liman *et al*. (2011) investigated that SPC concentration 4.0 mg/ml showed a high inhibition on acid production of *S. sobrinus 6715* and the corresponding ΔpH was 1.43±0.02. The result (table 1) showed that the SPC dimers exhibited stronger capacity of inhibiting acid production than SPC.

All these findings indicated that SPC dimers had potential to be used as anticaries preventive medicine due to its strong capacity of antigrowth and antiacid. For further studies of the GPC-3 separated by Toyopearl HW-40(s) gel column were needed. Accordingly, the relationship between the constituent of SPC and the anticaries effect of *S. sobrinus 6715* could be acquired.

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