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Solubility study of phytochemical cross-linking agents on dentin stiffness

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Abstract

Objectives—The effects of interactions between cross-linking proanthocyanidins (PA) in polar solvents and type-I collagen of demineralized dentin was investigated.

Methods—Three PA-rich extracts, two from grape seed (GSEP and GSES) and one from cocoa (COE), were dissolved (water, ethanol:water and acetone:water) and analyzed for their ability to increase the modulus of elasticity of demineralized dentin. Sound dentin beams (0.5 X 1.7 X 7 mm) were fully demineralized and divided into 12 groups according to the type of cross-linking agent and solvents used. Specimens were immersed in the respective solutions and tested at baseline, 10, 30, 60, 120 and 240 min.

Results—The elastic modulus (EM) of dentin was significantly increased by the PA treatment regardless of time ($p < 0.05$ for all times). The extracts showed different solubility in different solvents. GSEP showed the highest increase in EM when diluted in distilled water and acetone at all exposure times. Both GSEs showed superior results when diluted in distilled water and after 4 hours of treatment, while COE produced strongest enhancement when dissolved in ethanol:water.

Conclusions—The results indicates that herbal extraction process and other pharmacognostic parameters have an important influence on extract solubility as well as constitution and, consequently, on the PA-dentin matrix interaction.

Keywords

Dentin collagen; Cross-linkers; Polar systems; Proanthocyanidin; Hansen solubility parameters

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Introduction

Proanthocyanidin (PA) are an important class of secondary metabolites in higher plants and belong to the category known as condensed tannins [1]. PA are polyphenolic natural products composed of flavan-3-ol subunits linked mainly through C4–C8 (or –C6) bonds [2]. Recently, PA have received considerable attention in the fields of nutrition, health, and medicine due to their physiological activities, such as antioxidant capacity [3], anti-microbial effects [4], anti-inflammatory properties [5], effects on cardiovascular diseases [6], anti-allergic activity [7], and enzymes inhibitory activity against, e.g. phospholipase A2, cyclooxygenase and lipoxygenase [8]. Cocoa beans and grape seeds are well-known sources of PA [9].

While PA inhibit many enzymes, they have specifically been shown to facilitate the enzyme proline hydroxylase. The hydroxylation of proline is an essential step of the collagen biosynthesis [10]. PA were also known to stabilize and increase the cross-linkage of type-I collagen fibrils [11]. Type-I collagen is the predominant component of extracellular matrix and constitutes about 20–25% of total body protein. Most connective tissues such as tendon, skin, blood vessels, bone and dentin are mainly composed of type-I collagen [12].

Dentin type I collagen plays a number of structural roles such as scaffold for mineralization and viscoelasticity by forming a rigid, strong, space-filling biomaterial [13]. Inter-molecular cross-links are the basis for stability, tensile strength and viscoelasticity of the collagen fibrils [14]. Since the dentin collagen matrix is a vital component of the restoration of missing tooth structure, a stronger and more stable collagen is desirable for current restorative procedures, such as adhesive restorations. Previous study has demonstrated that a water-based PA-based agent increases the mechanical properties of demineralized dentin [15].

Proanthocyanidin are solids that represent complex mixture of compounds. Adequate design of processes and products that involve these phenolic compounds, requires knowledge of their physicochemical properties, particularly their solubility in different solvents and solvent systems. In this work, the aqueous solubility of some natural phenolic compounds (grape seed extract and cocoa extract) is addressed. To describe solubility behavior (δ_1), Hansen uses three solubility parameters (HSPs) [16]: atomic dispersion London forces (δ_d^2), permanent dipoles (polar interaction) (δ_p^2) and hydrogen bonds (δ_h^2). Recently HSPs have been extended to interpret the solubility of compounded materials such as high polymer materials, drugs and bio-ingredients of plants in various solvents [17]. The parameters follow the rule that the smaller the $\Delta\delta_{i,j}$ (mutual solubility between a solute, i , and a solvent, j), the greater the affinity between solute and liquid.

The present study aims to evaluate the effect of three PA-based collagen cross-linking agents on the modulus of elasticity of demineralized coronal dentin using different solvents (water, acetone and ethanol) and exposure times. The tested null hypothesis is that the use of collagen cross-linkers would not affect the elastic properties of demineralized dentin when compared to a non-treated group, regardless of the type of solvent and exposure time.

Materials and Methods

Proanthocyanidins based solutions

Various solvents (aqueous ethanol, aqueous acetone, distilled water) were used to prepare PA based solutions. All chemicals (Ethyl alcohol anhydrous, $\geq 99.5\%$ and Acetone, $\geq 99.9\%$, Sigma-Aldrich, St. Louis, MO) were used without further purification. The pH of the slightly acidic solutions was adjusted to 7.2 using NaOH. After pH adjustment, the solutions were filtered through paper filter n°6 (Whatman, London, England). The concentrations used were selected according to previous studies [15,18]. The treatment groups were as follows:

1. 6.5% (w/v) GSEP (*Vitis vinifera*, gold grape seed extract, Lot 13682503-01 Mega-Natural, Madera, CA, USA) in solutions.
2. 6.5% (w/v) GSES (*Vitis vinifera*, dried extract of grape seed, Lot 84277, Sanrisil, Sao Paulo, Brazil) in solutions.
3. 6.5% (w/v) COE (*Theobroma cacao*, polyphenol extract, Lot CMIE-7LJJKF-Foratero, Barry Callebaut, Lebbeke-Wieze, Belgium) in solutions.

Extracts were dissolved in the following solutions:

- A. 100% water solution (distilled water, DW).
- B. Ethanol:water solution (50:50, ET).
- C. Acetone:water solution (30:70, AC).

Neat solvents and solvents mixtures were used as negative controls.

Preparation of collagen samples

The use of eighteen sound human third molars as study material was approved by the Institutional Review Board Committee of the University of Illinois at Chicago under protocol no. 2009-0198. The teeth were kept frozen for no longer than 6 months, thawed, cleaned of adhering soft tissues, and the occlusal surfaces were ground flat with # 320 grit silicon carbide abrasive paper (Carbimet Discs, Buehler, Lake Bluff, IL) under running water to flatten the occlusal surface by cusp removal to enable more accurate sectioning of samples. The root portion was sectioned 1 mm below the CEJ and discarded.

Teeth were sectioned into 0.5 ± 0.1 mm thick disks in the mesio-distal direction using a slow speed diamond wafering blade (Buehler-Series 15LC Diamond) under constant water irrigation. The disks were further trimmed using a cylindrical diamond bur (# 557D, Brasseler, Savannah, GA) in a high speed handpiece to yield rectangular beams (0.5 mm thick $\times$ 1.7 mm wide $\times$ 7.0 mm length). A dimple was made at one end of the surfaces for orientation to allow repeated measurements to be performed on the same surface. Specimens were immersed in 10 % phosphoric acid solution (LabChem, Pittsburgh, PA) for a period of 5 h for complete demineralization and thoroughly rinsed with distilled water for 10 min.

Mechanical properties assessment

An aluminum ally flexural fixture with 2.5 mm span was glued to the bottom of a glass Petri dish. Specimens were tested in 3-point bending while immersed in liquid using a 1 N load cell mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) at crosshead speed of 0.5 mm/min.

Since the actual strain of the specimen was not measured with an extensometer or strain gauge, but only inferred from the displacement of the crosshead, the specimen stiffness values are termed “Apparent elastic modulus”. Displacement (D) during compression was displayed in millimeters and calculated at a maximum strain of 3% using the following formula [18]:

$$D = \epsilon L^2 / 6T$$

Where ϵ is strain, L is support span, and T is thickness of the specimen. The Apparent elastic modulus (E) of the specimens was expressed in MPa (Mega Pascal) and calculated using the following formula:

$$E=PL^3/4DbT$$

Where P is the maximum load, L is the support span, D is the displacement, b width of the specimen, and T is the thickness of the specimen.

The groups were: GSEP, GSES, COE dissolved in DW, ET, AC solvent systems, or only the solvent systems used as control groups. Specimens were immersed in water for baseline measurements and then in their respective solutions for cumulative exposure time. Time periods studied were: baseline, 10 min (t_{10}), 30 min (t_{30}), 60 min (t_{60}), 120 min (t_{120}) and 240 min (t_{240}). All measurements were performed with specimens immersed in distilled water. The data was collected and statistically analyzed using a General Linear Model in SPSS program (version 15.0) for ANOVA. Two-way ANOVA for each time period was computed to assess the effects of the treatment and solvent.

Calculation of Hansen Solubility Parameters (HSPs)

The total cohesive energy density (δ_i) is approximated by the sum of the energy density required to overcome atomic dispersion London forces (δ_d^2), forces between permanent dipoles of adjacent molecules (polar interaction) (δ_p^2), and to break hydrogen bonds (exchange of electrons, protons donors/acceptor) between molecules (δ_h^2). The solubility parameters for the solvents were calculated regarding their molar volumes and also their concentrations when mixtures, according to Hansen (2000) [16]. The mutual solubility between a solute_i and a solvent_j, was quantified, when not found in the literature, by the following parameter [16]:

$$(\Delta\delta_{i,j})^2 = [4(\delta_d^i - \delta_d^j)^2 + (\delta_p^i - \delta_p^j)^2 + (\delta_h^i - \delta_h^j)^2]^2$$

Results

Table 1 shows means and standard deviations for all treatment groups, while Table 2 presents *p-values* for all factors and interactions studied.

The interaction between treatment and solvent was statistically significant for all time periods (baseline $p = 0.033$, and for all exposure times $p < 0.005$), except for 10 min evaluation ($p = 0.062$). Except for baseline ($p = 0.497$), all treatment periods with the PA-based extracts increased the Apparent elastic modulus of dentin matrix ($p < 0.001$). Variations of solvents did not affect the stiffness of the samples at 10 (t_{10}), 120 (t_{120}), and 240 (t_{240}), min cumulative exposure times, ($t_{10} = 0.207$; $t_{120} = 0.106$; $t_{240} = 0.346$).

Figure 1 illustrates the behavior of the solvents in the experimental and control groups. COE had a more homogenous behavior when compared to the other treatment groups, establishing a closer relationship with the ethanol-water system. Generally, both groups treated with GSE showed stronger effects when dissolved in water, while for acetone-water solutions higher stiffness increase was seen only at 60 minutes evaluation.

HSPs were calculated for common compounds containing PA-rich extracts and they are listed on Table 3. The best solvent was 30% aqueous acetone which showed lower $\Delta\delta_{i,j}$ values, followed by 50% aqueous ethanol. Neat water showed the highest $\Delta\delta_{i,j}$ values, indicating an essential lack of affinity between solutes and solvent.

Discussion

The inherent strength of type I collagen, the major structural component of connective tissue, is derived from the extra-cellular formation of covalent intermolecular cross-links. Light and Bayle (1979) proposed that the increased stability of mature collagen was due to the presence of multivalent cross-links, forming a system of lateral and transverse cross-links in the fibril [19].

After the treatment with PA-based treatments/extracts, a great and consistent increase in the Apparent elastic modulus of dentin matrix was observed, despite the type of solvent system and exposure time (Table 2). Therefore, the null hypothesis must be rejected. Although all tested solutions showed increased stiffness values when compared to control groups, the solvent systems behaved differently for each extract. Both grape seed extracts revealed higher solubility in neat water. Cocoa extract treatment resulted in higher values of stiffness when an ethanol-water solution was used, followed by acetone-water solution.

PA are polyphenolic phytoconstituents with chemical structures based on flavonol-3-ol monomers. Their condensation to dimers, trimers, and higher oligomers yields the oligomeric PA compounds (also known as Oligomeric Proanthocyanidins, OPCs). In addition to different linkages of the monomeric matrices (i.e., <4-8> or <4-6>, among other single and double C-C linkages) the flavone skeleton can bear various substitutions patterns and differ in stereochemistry (i.e. catechin vs. epicatechin, gallocatechin vs. epigallocatechin). As a result, PA are typically very complex natural products to analyze and provide a phytochemical challenge [20]. It is reasonable to predict that the variation of chemical structure and overall composition affects the solubility characteristics of these natural products.

Some of the different phenolic compounds known to be contained in a grape seed extract show only minor variations of their structures, and consequently the δt values are closely related, as shown in Table 3. According to the HSP model, values on Table 3 classify the phenolic mixture as a polar fraction and show that acetone-water mixture is the best extraction solvent both for monomeric and oligomeric and polymeric PA from grape seed. Based in the HSP, water alone could not dissolve the large polymers of grape seed. However there are limitations when predicting OPC solubility in aqueous mixtures. As ethanol or acetone is added to water, the entropy of mixing increases and the structure of the solvent mixture become less ordered, favoring the interaction of the solute with the solvent mixture [21]. While cocoa polyphenols are similar to those in grape seed, the grape seed tannins consist of a very complex mixture of oligomers and polymers [22]. The relative simplicity in cocoa's PA composition could explain its chemical and physical properties, reactivity and solubility behavior. To our best knowledge, cocoa extract solubility parameters are not available in literature.

The primary distinction between the various PA-rich plant extracts that are available on the market today is their underlying extraction process, representing one of the key pharmacognostic parameters. The sourcing of start material and the manufacturing process have profound effects on the composition, potency and PA content of resulting extract. The grape seed extract from Mega-Natural contains approximate 95% PA and represents a hot water extract. GSE from Sanrisil contains approximately 13% PA and represents a 20% ethanol:water extract, whereas the cocoa was obtained by extraction with 30% acetone:water, to yield approximately 45% PA content (all content information was provided by the manufacturer's; independent analysis is subject of ongoing work). The superior solubility in less polar solvents such as ethanol and acetone was confirmed in this study. This shows that, the extraction process of commercial extracts seems to influence the solubility of the extracts more than established solubility parameters of isolated polyphenolic constituents.

Type-I collagen molecules are presented in a triple helices (roughly 280 nm long) form, a staggered structure within the fibrils with a periodicity of approximately 67 nm. Both intra- and inter-chain hydrogen bonding within the triple helices has long been thought to play an important role in forming the structure of collagen molecules [23]. The removal of associated water through chemical dehydration, i.e. replacement with different polar solvents such as methanol, acetone and ethanol, causes shrinkage of collagen matrix in demineralized dentin [24,25], and can lead to higher tensile modulus and ultimate tensile strength (UTS) [26,27]. Such stiffening and strengthening of dentin collagen has been attributed to increased levels of interpeptide hydrogen bonding between adjacent collagen fibrils as the collagen structure was disrupted by the substitution of water for solvent molecules. The amount of shrinkage and the increased modulus of elasticity and UTS were found to be inversely proportional to the hydrogen bonding ability of the solvent, as measured by the HSP for hydrogen bonding, δ_H [16]. With its relatively higher δ_H value, water can plasticize collagen by breaking the interpeptide hydrogen bonds, leading to a lower modulus and strength [26]. The distilled water control group showed only minor changes in stiffness. In previously published studies, control specimens were exposed to dehydrating polar solvents [26,27]. In the current study, all control measurements were done in water which is known to reverse increases in matrix stiffness caused by water-free polar solvents [26]. In the current study, all control group solvents were water-based. No pure (i.e. neat) solvents were used, which differs from previously published studies [26,27].

HSPs are presented in literature for plant derived products [21]. In the present study, the OPC extraction method used by the manufacturer had apparent influence on the solubility parameters not only tannin monomers, but also the oligomers of the PA complex. PA-rich extracts may consist of hundreds of known, and perhaps thousands of unknown, naturally occurring and biologically active substances other than PA, such as quercetin, myricetin, ferulic acid, caffeic acid and/or coumaric acid. Therefore, the goal of ongoing efforts is to characterize the full spectrum of polyphenolic compounds contained in Grape seed and cocoa seed products in order to establish a correlation between their phytochemical contents and their stiffness enhancing properties.

When designing a biomaterial, and depending on the specific application, the properties of collagenous matrices can be tailored by cross-linking. The data obtained in the present study provides rationales for choosing a solution of a natural cross-linking agent that will target the desired physical properties of the collagen matrices. The results lead to a preliminary understanding of the effect of both the natural cross-linking extracts and the employed solvents on collagen properties, and are potentially valuable in the design of a stronger dentin matrix substrate less prone to degradation. Other factors such as solution temperatures and longevity of the cross-linked collagen products have to be evaluated.

Conclusions

The nature of the plant extracts and the method of collagen cross-linking influences the physical properties of dentin matrices. Natural extracts from grape seed and cocoa seed were found to be effective in improving the stiffness of demineralized dentin. Factors such as solubility of the products were found to have significant influences on the mechanical properties of dentin matrices and their overall cross-linking potential.

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Figure 1.

Figure 1a. - Graph of the Apparent elastic modulus (MPa) values of demineralized dentin treated with grape seed extract from Mega-Natural; DW-distilled water; ET-ethanol-water; AC- acetone-water.

1b – Graph of the Apparent elastic modulus (MPa) over time of demineralized dentin treated with grape seed extract from Sanrisil; DW-distilled water; ET-ethanol-water; AC- acetone-water.

1c – Graph of the Apparent elastic modulus (MPa) over time of demineralized dentin treated with cocoa extract; DW-distilled water; ET-ethanol-water; AC- acetone-water.

1d – Graph of the Apparent elastic modulus (MPa) over time of demineralized dentin non treated; DW-distilled water; ET-ethanol-water; AC- acetone-water.

NOTE: different scales in each graph.

Table 1

Repla for Apparent modulus of elasticity [means (standard deviations)] of demineralized dentin matrices following different treatments, solvents, and exposure times.

		Modulus of elasticity (MPa)					
Treatment	Solvent	Exposure Time					
		baseline	10 min	30 min	60 min	120 min	240 min
GSEP	DW	6.59 (2.59)	39.64 (17.95)	61.24 (27.34)	73.65 (36.96)	92.85 (50.24)	109.85 (53.18)
	ET	6.71 (2.61)	26.69 (11.41)	30.99 (22.84)	39.33 (14.39)	56.86 (21.20)	72.12 (25.07)
	AC	6.20 (2.35)	33.58 (15.49)	57.17 (25.22)	77.75 (26.64)	77.67 (29.10)	79.32 (29.81)
GSES	DW	6.69 (1.49)	17.85 (5.65)	27.32 (13.75)	32.69 (18.65)	46.01 (26.26)	54.74 (33.75)
	ET	5.93 (1.75)	12.29 (4.79)	15.52 (5.98)	20.01 (8.22)	24.54 (10.69)	33.53 (14.00)
	AC	7.99 (1.86)	18.80 (3.15)	25.59 (6.58)	37.43 (8.30)	37.84 (8.66)	51.54 (13.24)
COE	DW	6.68 (2.25)	21.84 (12.95)	28.25 (17.47)	34.30 (21.70)	39.93 (23.04)	53.47 (28.54)
	ET	8.73 (3.15)	25.78 (12.72)	33.90 (15.45)	43.71 (27.30)	55.84 (36.08)	79.97 (54.42)
	AC	6.28 (3.08)	18.82 (9.06)	24.20 (11.54)	33.50 (17.09)	46.24 (17.33)	58.39 (25.84)
Control	DW	6.99 (1.85)	5.85 (1.47)	6.23 (1.27)	6.01 (1.17)	6.19 (1.42)	6.19 (1.51)
	ET	5.74 (1.98)	5.76 (2.03)	5.42 (1.76)	5.19 (1.76)	5.23 (1.59)	5.03 (1.48)
	AC	6.76 (2.05)	6.85 (2.00)	7.21 (2.26)	7.17 (2.33)	6.81 (1.87)	6.95 (1.70)

DW- distilled water; ET- 50% ethanol-water solution; AC- 30% acetone-water solution

Table 2Two-Way ANOVA between-subjects effects results-(*p-value*)

	Baseline	10 min	30 min	60 min	120 min	240 min
Treatment	0.497	0.000 *	0.000 *	0.000 *	0.000 *	0.000 *
Solvent	0.989	0.207	0.005 *	0.009 *	0.106	0.346
Treatment * Solvent	0.033 *	0.062	0.000 *	0.001 *	0.021 *	0.013 *

* statistically significant ≤ 0.05

Table 3

Hansen solubility parameters. The values of V_m , δ_d , δ_p , δ_h and δ_t for the phenolic compounds were described by Savova et al. (2007) [21]. $\Delta\delta_{i,j}$ were calculated according to Hansen Solubility Parameters: a user's handbook [16].

	$V_m \text{ Cm}^3\text{mol}^{-1}$	$\delta_d \text{ MPa}^{1/2}$	$\delta_p \text{ MPa}^{1/2}$	$\delta_h \text{ MPa}^{1/2}$	$\delta_t \text{ MPa}^{1/2}$	$\Delta\delta_{i,j} \text{ H}_2\text{O}$	$\Delta\delta_{i,j} \text{ 50\% ethanol/water}$	$\Delta\delta_{i,j} \text{ 30\% acetone/70\% water}$
Catechin	266	13.7	4.5	19.7	24.4	27.79	16.53	15.93
ECG	385	13.8	3.8	19.7	24.4	28.27	17.09	16.32
EGC	273	13.6	4.7	21.2	25.6	26.53	15.42	14.75
EGCG	392	13.8	4.0	20.8	25.3	27.37	16.28	15.40
Dimmers	518	13.4	3.3	19.9	24.2	28.46	17.34	16.69

EGC-epigallocatechin; ECG-epicatechin gallate and EGCG-epigallocatechin gallate.

$|\delta^2_d|$ - atomic dispersion London forces; δ^2_p - forces between permanent dipoles of adjacent molecules (polar interaction); δ^2_h - hydrogen bonds (exchange of electrons, protons donors/acceptor) between molecules.