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Centrifugal partition chromatography enables selective enrichment of trimeric and tetrameric proanthocyanidins for biomaterial development

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ABSTRACT

Proanthocyanidins (PACs) find wide applications for human use including food, cosmetics, dietary supplements, and pharmaceuticals. The chemical complexity associated with PACs has triggered the development of various chromatographic techniques, with countercurrent separation (CCS) gaining in popularity. This study applied the recently developed DESIGNER (Depletion and Enrichment of Select Ingredients Generating Normalized Extract Resources) approach for the selective enrichment of trimeric and tetrameric PACs using centrifugal partition chromatography (CPC). This CPC method aims at developing PAC based biomaterials, particularly for their application in restoring and repairing dental hard tissue. A general separation scheme beginning with the depletion of polymeric PACs, followed by the removal of monomeric flavan-3-ols and a final enrichment step produced PAC trimer and tetramer enriched fractions. A successful application of this separation scheme is demonstrated for four polyphenol rich plant sources: grape seeds, pine bark, cinnamon bark, and cocoa seeds. Minor modifications to the generic DESIGNER CCS method were sufficient to accommodate the varying chemical complexities of the individual source materials. The step-wise enrichment of PAC trimers and tetramers was monitored using normal phase TLC and Diol-HPLC-UV analyses. CPC proved to be a reliable tool for the selective enrichment of medium size oligomeric PACs (OPACs). This method plays a key role in the development of dental biomaterials considering its reliability and reproducibility, as well as its scale-up capabilities for possible larger-scale manufacturing.

1. Introduction

The concept of chemical subtraction entails the selective removal of targeted component(s) from a complex mixture or an extract. It was first developed in 2008 and experimentally demonstrated by the removal of benzoic acid from cranberry juice fractions using countercurrent separation (CCS) [1]. Due to the broad applicability of this technique in the field of natural product research, the concept was further developed to encompass both the selective enrichment and/or the depletion of extracts, guided by the intended biological application. The term DESIGNER (Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources) was, thus, coined and the methodology was demonstrated for bioactive prenylated flavonoids from the extract of *Humulus lupulus* [2]. The current study demonstrates a new application of the DESIGNER methodology for the preparation of proanthocyanidin based biomaterials (Fig. 1) that were prepared for dental hardtissue applications.

Proanthocyanidins (PACs) are oligomers of monomeric flavan-3-ols. PACs are ubiquitously present and structurally complex metabolites that plants synthesize as a predatory defense mechanism. Many food plants such as grape seeds, green tea, mangosteen pericarp, cinnamon bark, cocoa seeds, and cranberries are particularly rich in PACs. The increasing number of reports on the in vivo and in vitro bioactivities of PACs is primarily contingent upon their anti-oxidant and anti-inflammatory activity, as well as their anti-infective potential [3–6]. In fact, most recently, FDA approved the plant-based oligomeric PAC (OPAC) mixture obtained from

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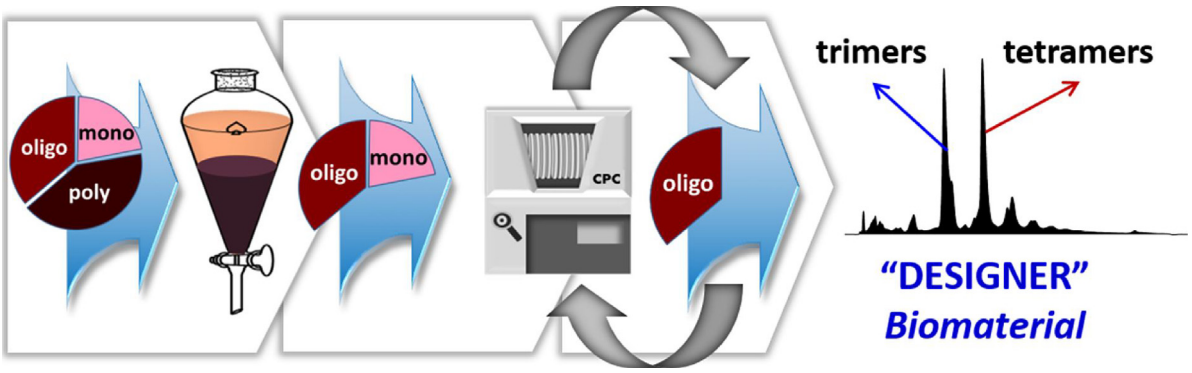

Fig. 1. Application of the DESIGNER concept for the selective enrichment of PACs. Schematic representation of the methodology used for the preparation of trimer and tetramer enriched DESIGNER material from various proanthocyanidin-enriched plant sources.

Croton lechleri, Fulyzaq^{R} (crofelemer, now sold under the brand name Mytesi^{TM}), for the alleviation of HIV-associated diarrhea (*FDA* Fulyzaq^{R} *approval*) [7,8].

Recent efforts aimed at the development of PAC-based biomaterials have resulted in several chemical separation, optimization, and dentin biomechanical evaluation studies aimed at prolonging the lifespan of composite-based dental restorations [9]. Through recently performed invitro studies on dentin, it was observed that the OPACs, especially trimers and tetramers, as well as their galloylated analogues, are potent dentin biomodifiers [10–12]. Certain OPACs are capable of structurally modifying dentin via inter-molecular and inter-microfibrillar collagen cross-linking. Optimum molecular size (degree of polymerization) and shape (relative/absolute stereochemistry) of the OPACs are the most important factors for this biomimetic property. Thus, the present study focuses on the application of CPC methodology for the preparation of PAC trimer and tetramer enriched (DESIGNER) biomaterials.

The structural possibilities for OPACs increase exponentially based on the variable monomeric units, the type (A vs. B) and position of the interflavan linkages (IFLs), the degree of polymerization (DP), and the inherent stereochemistry of the monomers (Fig. 2). In consequence, separation of OPACs from the already chemically complex plant extracts has been a significant chromatographic challenge, even with the development of advanced analytical techniques [13]. Despite the apparent similarities of the basic OPAC skeletons, their structural differences deceive the untrained human eye in both 2D and 3D molecular representations, but, in fact, the variances are major and give rise to distinct chromatographic fingerprints and biological potential for each plant source. Consequently, specific chromatographic methods need to be adapted according to the starting material, despite the goal of separating the same general class of compounds. The chromatographic elution order of the different classes of PACs and the number of steps required to achieve the desired separation depends on the specific PAC chemistry of each plant source.

While hydrodynamic CCS methods employing HSCCC (high speed countercurrent chromatography) have been used frequently [14–18], their success depends on the specific type of the target molecules. For instance, HSCCC has enabled successful separation of green tea polyphenols that mainly represent mixtures of the flavan-3-ol monomers, catechin, epicatechin, epigallocatechin gallate, and epicatechin gallate, along with galloylated dimers [15]. Separation of grape seed extract by HSCCC yields relatively poor resolution of the dimers and leads to co-elution of all higher oligomers [18]. In fact, as documented in the literature, PAC research hardly extends beyond dimers, owing to the increasing complexity of the higher order oligomers with regards to chromatography as well as structural analyses. The current study presents a scheme for preparative separation that yields the desired (most bioactive) DP 3 + 4 cuts from the PAC-rich extracts using source-dependent, minor solvent system modifications and employing CPC methodology. The effect of step-wise enrichment of trimers and tetramers from grape seed and cocoa extract on dentin biomodification is also demonstrated using dentin stiffness as the means of evaluation of biomechanical enhancement.

2. Experimental

2.1. Materials

Grape seed extract (*Vitis vinifera*, VV) was kindly donated by Polyphenolics MegaNatural Gold Grape Seed Extract, Madera, California, USA (No. 206112508-01/122112505-01). Cocoa extract (*Theobroma cacao*, TC) was obtained from Barry Callebaut Ltd., NCE- PO804-WW41 [polyphenol extract], Meulan, France, (No. 700900001). Pine bark extract (*Pinus massoniana*, PM) was obtained from Xi'an Chukang Biotechnology Co. Ltd., China (No. PB120212). Cinnamon stem-bark powder (*Cinnamomum verum*, CV) was purchased from Oregon's Wild Harvest, Sandy, Oregon, USA (No. CIN-07011p-OMH01) and extracted in-house using 70% acetone, and the extract was dried and lyophilized. All solvents were of analytical grade and obtained from Fisher Scientific (Fair Lawn, NJ, USA) or Sigma-Aldrich (St. Louis, MO, USA).

2.2. Instruments

A centrifugal partition chromatography (CPC) extractor, SCPC-250-B (Armen Instrument Gilson Inc. SAS, France), was used. Diol-HPLC profiles were acquired on a Waters 600 HPLC (Waters, USA) instrument using a Develosil 5 μm Diol 100A 250 × 4.6 mm column (Phenomenex, Torrance, CA, USA) equipped with a Waters 2996 PDA detector.

2.3. Preparation of trimeric and tetrameric PAC enriched fractions

A three-step separation scheme was employed beginning with the depletion of polymeric PACs, followed by a monomer knock-out step and a trimer and tetramer enrichment in the final step (Fig. 1). Slight modifications were made for individual methods based on the plant source, level of complexity and type of PAC chemistry/composition, as indicated below. A flow rate of 30 (for TC, PM and CV) or 35 ml/min (for VV) and rotation speed of 2500 rpm was used throughout. All the CPC separations were performed at room temperature without additional temperature control. An analytical Diol HPLC column was used for the monitoring of CPC fractions. The HPLC mobile phase consisted of acetonitrile/acetic acid (98/2) as solvent A and methanol/water/acetic acid (95/3/2) as solvent B. A

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