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Proanthocyanidin Metabolism, a mini review

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Abstract. There is emerging evidence suggesting that consumption of beverage and food rich in polyphenol may offer protective effects against various neurodegenerative, cardiovascular diseases and cancers. Proanthocyanidins (PACs) are one of the most abundant polyphenol in human diets, but also one of the least absorbed polyphenol mostly due to their size and structure complexity. PACs or condensed tannins are oligomers and polymers of monomeric unit flavan-3-ol (+)-catechin or (−)-epicatechin. To date, the absorption and metabolism of PACs are still remains largely unknown. The aim of this mini review was to highlight the absorption and metabolism of PACs, their effect in the gut and sample preparation for analysis. Ultimately, the potential bioactivities derived from the interaction between PACs metabolites and the gut microbiota warrants further investigation.

Keywords: Proanthocyanidins, phenolic acids, metabolism, colon

1. Introduction

Polyphenols are among the ubiquitous constituents of foods of plant origins and are widely distributed throughout the plant kingdom. Polyphenols can be categorized into different groups such as flavonoids, phenolic acids, stilbenes and lignans. The flavonoids consists of 2 aromatic rings (A and B) that are link with a heterocyclic five-membered ring that includes an oxygen atom (ring C), and divided into 6 subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins) [22]. Proanthocyanidins (PACs), a subclass of flavonoids, are among the most abundant polyphenol present in human diets. The dietary intake of PACs in the U.S. was estimated to be about 100 mg/d, falling into the following size categories (percentage of total), monomers (22%), dimers (16%), trimers (5%), 4–6 mers (15%), 7–10 mers (11%), and polymers (30%), and PACs can be found in various foods and beverages including grape seeds, pine bark, apple, peach, pear, berries, cocoa, tea, wine and beer [49]. PACs are considered one of the most complex subclass of flavonoids, consisting of monomers, oligomers or polymers of (−)-epicatechin or (+)-catechin and derived subunits. PACs that consists exclusively of (−)-epicatechin, (+)-catechin units, are known as procyanidins because only cyanidin is released in acid, and comprised the largest class of PACs, while those with gallocatechin units release delphinidin. The size or molecular weight of PACs can be expressed as mean degree of polymerization (mDP). The major constituents of grapes are monomeric (−)-epicatechin, (+)-catechin, epicatechin-3-O-gallate (more abundant in seeds than in skins) and (−)-epigallocatechin (only found in skins) [11]. Therefore, grape seed tannins are partially galloylated procyanidins. The dimeric procyanidins are often referred as B-type (B1, B2, B3 B4 and B5) or A-type (A1 and A2) and the trimeric procyandins as C-type (C1 and C2). The most ubiquitous PACs in foods are the B-type, formed by oxidative coupling between the (C4–C8) bond and to a lesser extent through a (C4–C6) bond. The less common is the A-type procyanidins which have an additional ether bond through a (C2-O-C7) bond or a less abundant (C2-O-C5) bond [48].

2. Absorption and metabolism data of oligomeric and polymeric PACs

PACs are known to be poorly absorbed in the gut due to their polymeric nature and high molecular

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weight. Thus, polyphenols are the only dietary antioxidants that are present in the colon at levels up to a few hundred micromoles per liter, given that vitamins C and E are absorbed through the upper intestines [22]. This limitation on their absorption may result in a direct beneficial activity in the gastrointestinal (GI) tract. Physiologically phenolics including PACs are treated as xenobiotics, and the small intestine is the primary site for glucuronidation, occurring in luminal part of the endoplasmic reticulum through uridine 5'-diphosphate glucuronosyltransferases (UGTs) while sulfation and methylation occur in the liver via cytosol sulfotransferases (SULT) and catechol-O-methyltransferase (COMT) [27]. Before entering the blood stream, the monomeric or dimeric flavan-3-ols are either absorbed in the small intestine or transported back to the liver and undergo phase II metabolism, producing glucuronide, sulfate and/or methylated metabolites. Through enterohepatic recirculation, these conjugated compounds may recycle back to the small intestines via bile excretion. It has been estimated that more than 90% of ingested polyphenols are not absorbed in the small intestine and thus, remain in the colon at high concentration [7]. In the colon, the unabsorbed compounds are extensively metabolized by gut microbiota to produce smaller molecules, including simple phenolic acids such as hydroxybenzoic acid, hydroxyphenylacetic acid, hydroxyphenylpropinoic acid, hyrdroxyphenylvaleric acid or hydroxycinnamic acids, with hydroxylation mostly occurring at meta position [1, 23]. These metabolites can be absorbed or may be conjugated in the liver before being eliminated in the urine. In some cases, the amount of the simple metabolites is significant, suggesting that these metabolites may have important functions [39].

Monomeric flavan-3-ols, including (+)-catechin and (−)-epicatechin, has been previously reported to be absorbed in humans and animals, either as parent compounds or conjugated metabolites following the consumption of dietary PACs [3, 50]. While studies of the metabolism and absorption of monomeric flavan-3-ols are numerous, investigations on the fate of oligomeric and polymeric PACs are controversial, being mostly focused on the fate of dimeric PACs. The quantification and identification of PACs dimers and trimers in plasma or urine following the ingestion of apples [37], cocoa [16, 46] and GSE [30, 35, 36] PACs has been demonstrated. The presence of these oligomeric PACs in plasma suggested that they were absorbed and metabolized similarly to monomeric flavan-3-ol. Low level of PACs dimer B2 has been reported in human urine and plasma after consumption of cocoa PACs [16, 46] while dimer B1 was detected in human plasma following ingestion of GSE PACs [35]. The absorption of dimers were estimated to be 100 fold lower than the monomer [16]. PAC dimers and trimers have been detected in the rats urine fed GSE [44]. Recently, Serra et. al have quantified and identified intact PACs dimers and trimers in rats plasma with the concentration ranging from 0.85 – 8.55 μM [36]. The absorption of A-type procyanidins were less studied compared to the B-type. Nevertheless, it has been reported that A-type procyanidins (A1 and A2) were absorbed from small intestines in rats and they were better absorbed than dimer B2 [1, 2]. Future study should be focused on these A-type procyanidins since it has been shown to have protective effects against cardiovascular diseases [21] and an effective uropathogenic bacterial anti adhesion activity [17]. It has been reported that apple PAC oligomers up to pentamers were detected in rat plasma 2 h after oral administration, [37] however, the administration dose was rather high, 1 g/kg body and may not be physiologically relevant. Studies from Rios et.al showed that cocoa PACs were stable in the stomach and reached in the small intestine, and available for absorption or metabolism [32]. A recent feeding study of apple juice to ileostomists have shown that most polyphenols remained unchanged at the large intestine but extensively metabolized by gut microbiota in the colon to various phenolic acids [19]. Due to low absorption of intact PACs in the human colon, microbial metabolism is likely has a major role in colonic health and the potential biological effects are attributed to these gut microbiota metabolites. (reviewed in [8]) In *in vitro* studies, it has been reported that human gut microbiota degrades PACs to low molecular weight phenolic compounds, [9] suggesting that absorption of the PACs parent compounds is unlikely. Urpi-Sarda et al. have detected various microbial phenolic metabolites and phenylvalerolactones in human and rats urine after cocoa PACs consumption [46].

3. Colonic microbial metabolism of PACs

There is no consensus on the absorption and metabolism of PACs thus far, although colon is

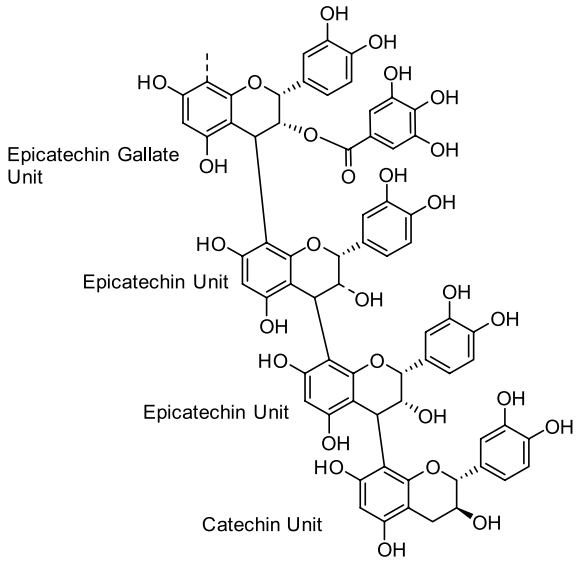

Fig. 1. Sample grape proanthocyanidin tetramer shown with bond extension to additional subunits.

being recognized as an important biotransformation site of these PACs by the gut microbiota. Therefore, the PACs are degraded by either intestinal enzymes or the gut microbiota before they can be absorbed. The potential bioactivities of PACs are attributed to the colonic degradation products, including phenolic acids and valerolactones. Numerous *in vivo* studies have showed the presence of microbial derived phenolic metabolites in urine after intake of PACs-rich diets. Some of the main urinary microbial phenolic acids found were mono- and di-hydroxy phenylpropionic acids and phenylacetic acids, along with hydroxyhippuric acids [27]. Gonthier et al. found an increase in 3-hydroxyphenylacetic and 3,4-dihydroxyphenylacetic acids in urine after the intake of procyanidin B3 in rats [13]. Similarly, Urpi-Sarda et al. reported a significant increase in the urinary level of 3-hydroxyphenylacetic, 3,4-dihydroxyphenylacetic acids and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone after regular consumption of cocoa [46]. The main microbial metabolites detected in rat urine after fed with red wine polyphenols were 3-hydroxyphenylpropionic acid, 3-hydroxybenzoic acid and 3-hydroxyhippuric acid [12]. The identification of bacteria to catabolize PACs should be further evaluated for a better understanding of the role of PACs-rich diets and relationship of gut microbiota in promoting overall gut health.

4. Effect of PACs in the gut

In general, PACs are thought to be poorly absorbed and their health effects may not require direct absorption in the gut. The large PACs (oligomeric or polymeric) can regulate cell signaling pathways by interacting with cell membrane proteins [5], and protecting intestinal barrier integrity through different mechanisms of action including their antioxidant capacity and anti-inflammation activity.

A study has reported that intact parent PACs (dimer-hexamer) were detected in the colonic rat feces after ingesting GSE PACs and these dietary components may contribute to the colon health [6]. This is also in line with a recent finding that detected microbial phenolic metabolites in feces, cecal content and colonic tissue following intake of PACs-rich diets [43].

There is mounting evidence from *in vitro* and *in vivo* studies of potential health benefits of PACs consumption linked to protection against colorectal cancer (CRC) [20, 38, 47]. Currently, CRC is the third most diagnosed cancer in the US. Epidemiological and clinical studies have reported that high intake of fats and red meat increases the risk of colorectal cancer (CRC) [41]. The consumption of fruits and vegetables, however, is linked to the decrease risk of CRC and may be due to their high content of flavonoids [41, 42]. Among the flavonoids, PACs ($\geq$2 subunits) consumption was found to be inversely related to the decrease risk of CRC in an Italian population [33]. The health effects may be attributable not only from the intact PACs but also their microbial phenolic metabolites that are readily absorbed. Thus, a better understanding of the metabolic fate of oligomeric or polymeric PACs in the colon is essential to understand their potential colonic health benefits.

5. Challenges of analysis of PACs

PACs are susceptible to oxidation and highly reactive natural products. Their diverse structure and high molecular size make the identification and quantitation of these compounds challenging. Chromatographic methods with combinations of various mass-spectrometry techniques have been the forefront application used in identification and quantification of metabolites in biological samples.

Sample preparation is a key step during method development, particularly working with complex

biological matrices since the clean-up procedures can greatly affect the results. A thorough sample preparation is needed to minimize matrix effects and increase instrument sensitivity when quantifying compounds of interest by mass-spectrometry. Different biological matrices may require different clean up procedures to remove interference compounds including protein, carbohydrate, salt, organic and inorganic compounds.

Protein precipitation is one the simplest way to remove proteins in biological samples and it has been used in the first step of sample preparation [29]. Protein precipitation was carried out by adding organic solvents such as acetonitrile to the sample, a solvent that dissolves PACs quite well.

One of the most common cleanup methods used by many investigators was solid phase extraction using C_8 or C_{18} SPE cartridges [29, 36]. During feces sample preparation, 50% aqueous methanol has been shown to be the best extraction solvent and recovery as elution solvent in C_{18} solid phase extraction (SPE) cartridges for GSE PACs [6]. Therefore, the feces sample was cleaned up and extracted by liquid-solid extraction and the extracts were subjected to SPE. Recently, Oasis® hydrophilic-lipophilic balanced (HLB) cartridges have been widely used for polyphenolic compounds extraction [43]. The stationary phase of HLB is a reverse-phase sorbent, a polystyrene divinylbenzene copolymer and it is suitable for acids, bases and neutrals compounds. Additionally, it has been reported that 96-well plates were used for sample cleanup including plasma and urine [46]. Some of the advantages of using conventional SPE cartridges over 96-well plates were high throughput sample processing and less usage of organic solvents.

Besides SPE, liquid-liquid extraction can be used for sample extraction or as a cleanup method. This technique is suitable for lipophilic compounds extraction and solvents that commonly used were ethyl ether or ethyl acetate. High molecular weight polyphenolic compounds were extracted using aqueous organic solvent including methanol and 70% aqueous acetone. It has been reported that acidification of the extraction solvent can increase the extractability of PACs since acid helps to break the bonds between PACs and polar matrices [31].

The spike recoveries of phenolic metabolites in biological samples were varied and ranged from 80–100% in urine [46] to 50–115% in feces [6]. It has been observed that the higher the DP of PACs, the lower the spike recovery. These lower recovery rates are likely due to increasing adsorption of the larger oligomers to the complex matrix present in the feces [6].

Analytical separation and identification techniques for polyphenolic compounds includes thin layer chromatography (TLC), gas chromatography (GC) and/or liquid chromatography (LC) coupled to various mass spectrometry such as single quadrupole, triple quadruople, ion trap, time-of-flight (TOF) and nuclear magnetic resonance (NMR) [4, 15, 26, 29]. The chromatographic separations were mostly performed using HPLC while GC was used for separation of low molecular weight or volatile polyphenolic compounds [14]. A reverse phase-LC method was able to separate flavan-3-ol monomer up to trimers and DP >3 will elute as a broad and unresolved peak at the end of the run.

Nonetheless, normal phase liquid chromatography (NP-LC) coupled with a fluorescence detector was able to separate and quantify individual PACs up to decamers [4].

A soft ionization source such as electrospray ionization (ESI) was commonly used for identifying polyphenol metabolites. Besides ESI, other ionization sources including atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) has been used for detecting small metabolites [29]. Both positive $[M+H]^+$ or negative $[M-H]^-$ ion mode can be used, although negative ion mode yields ions more effectively, due to the weak acidic nature of PACs [15].

The application of LC-MS/MS for quantitation of polyphenol compounds have been shown by others [4, 30, 46]. For metabolite identification, LC-MS/MS operating in multiple-reaction monitoring (MRM) offered high selectivity and sensitivity allowing detection of metabolites in biological samples at low concentration.

6. Conclusions

The adsorption and metabolism of proanthocyanidins is only partially understood, largely due to the amphilic physical properties, the complex mixtures of the natural sources and very low adsorption rates. Consequently, analytical approaches to study these substances are complex, but highly sensitive LC-MS systems capable of ionizing high molecular weight compounds are making the questions more approachable. The other complicating factor is that since the

adsorption in the intestine is very ineffective, the compounds are largely metabolized in the colon, meaning that compounds may be important to gut chemistry, but also that the metabolites are highly fragmented by microbial action and thus more difficult to associate with the PACs. One of the most pressing questions is whether or not any PAC's remain intact in the colon, and if so, what is the concentration of these substances.

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