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Impact of condensed tannin size as individual and mixed polymers on bovine serum albumin precipitation

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ABSTRACT

Condensed tannins composed of epicatechin from monomer to octamer were isolated from cacao (*Theobroma cacao*, L.) seeds and added to bovine serum albumin (BSA) individually and combined as mixtures. When added to excess BSA the amount of tannin precipitated increased with tannin size. The amount of tannin required to precipitate BSA varied among the polymers with the trimer requiring the most to precipitate BSA (1000 μg) and octamer the least (50 μg). The efficacy of condensed tannins for protein precipitation increased with increased degree of polymerisation (or size) from trimers to octamers (monomers and dimers did not precipitate BSA), while mixtures of two sizes primarily had an additive effect. This study demonstrates that astringent perception is likely to increase with increasing polymer size. Further research to expand our understanding of astringent perception and its correlation with protein precipitation would benefit from sensory analysis of condensed tannins across a range of polymer sizes.

1. Introduction

Tannins are plant secondary metabolites that play a role in plant defence; mainly as a deterrent to herbivory (Hagerman & Butler, 1991; Harborne & Grayer, 1993), although there is some evidence of defence against microorganisms (Dixon & Lamb, 1990). Tannins also play an important role in a number of economically important areas of agricultural production; prevention of pasture bloat (Li, Tanner, & Larkin, 1996), the tanning of hides (Haslam, 1998) and in the mouthfeel of red wines (Gawel, 1998; Malien-Aubert, Dangles, & Amiot, 2002; Ribéreau-Gayon, 1982). In most of these systems, it is the ability of tannin to precipitate protein that underpins their function (Haslam, 1998). As a result the interaction of protein with tannin has attracted considerable research effort.

Tannins are broadly grouped into two classes; hydrolysable and condensed tannins, both derived from plants. Hydrolysable tannins, which occur widely in wood, are based on gallic acid and occur as aggregations of gallates, known as ellagitannins, or are comprised of gallic acid moieties bound to a central glucose, e.g. penatgalloyl glucose and known as gallotannins (Haslam, 1998). Condensed tannins are polymers of flavanol subunits and are common in soft plant tissues such as leaves and fruit (skin and seeds) (Haslam, 1998). Tannins are a heterogeneous mixture containing a range of different polymer sizes, subunit compositions, and subunit linkages (Haslam, 1998). In grape seed alone it has been estimated that there is in excess of 65532 unique chemical tannin structures (Adams & Harbertson, 1999).

Studies of tannin-protein interactions to date have given a strong indication that protein precipitation increases with increasing size (Porter & Woodruffe, 1984) and that the mode of interaction between tannins and proteins is a combination of hydrogen bonding and hydrophobic interactions (Charlton et al., 2002; Hagerman, Rice, & Ritchard, 1998). However, a limitation of all of the previous work was that owing to the complexity of isolating and purifying individual tannin polymers, these experiments have been conducted on mixtures of tannins with a range of polymer sizes and variable subunit composition (Porter & Woodruffe, 1984). The risk in using ill-defined mixtures and average polymer length to define tannin interactions and their correlation with astringency is the assumption that all tannins within a mixture interact with protein similarly.

There have been no studies to date that document the interaction of thoroughly characterised individual condensed tannin polymers with protein. Consequently, several essential aspects about how tannins interact with protein are not well understood. There have been no studies of tannin-protein interactions that directly compare the protein precipitation of individual condensed tannins of increasing polymer size to unequivocally demonstrate the

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relationship between polymer size and precipitation. The minimum molecular weight tannin required for protein precipitation has not yet been reported although it is thought that tannins less than tetramer do not precipitate bovine serum albumin protein (Adams & Harbertson, 1999). The impact on protein precipitation of structural features such as subunit composition is unclear as is the effect of different linkages (i.e. branched versus linear polymers).

To address these questions, a methodology that would allow isolation of individual polymers was required. Such a method was developed for the separation of cacao polymers (Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006). Condensed tannins from cacao are comprised of a single flavanol subunit, epicatechin, and can be readily separated up to octamer, although yields decrease with increasing polymer size. Using this method it has been possible to isolate sufficient quantity of pure polymers to determine the efficacy of tannin of different sizes for precipitating protein and the synergistic effect of these tannins in mixtures. Our hypotheses were that the efficacy of tannin for precipitating protein increases with size and the efficacy of polymer mixtures for precipitating protein is dependent upon polymer size.

2. Materials and methods

2.1. Chemicals

Raw unfermented organic cacao (*Theobroma cacao*, L.) seeds of Equadorian origin were obtained from Natural Zing (Mt. Airy, MD, USA). Acetonitrile, methanol, hexane and ethyl acetate solvents were purchased from Merck (Darmstadt, Germany). Bovine serum albumin, sodium dodecyl sulphate, sodium hydroxide, triethanolamine, hydrochloric acid, acetic acid, formic acid, ferric chloride, ammonium formate, Amberlite resins, phosphorous pentoxide, (−)-epicatechin and (+)-catechin were purchased from Sigma-Aldrich Pty. Ltd (Castle Hill, Australia). HPLC columns were purchased from Phenomenex (Torrance, CA, USA).

2.2. Cacao tannin extraction and isolation

Cacao seeds (500 g) were frozen with liquid nitrogen and ground in an electric coffee grinder to a 325 mesh (~44 microns particle size). Ground cacao was defatted with hexane in a Soxhlet extractor for 6 hours. Defatted cacao was air dried overnight in a fume hood. To extract cacao tannin, a 100 g sample of defatted cacao was extracted twice with 1 L of 70% aqueous acetone on an orbital mixer (250 rpm; 30 min). To remove solids, the sample was filtered under vacuum through a glass microfiber filter. The filtrate was rotary evaporated under reduced pressure (40 °C) to remove acetone. The sample was mixed twice with 500 mL of ethyl acetate to remove low molecular weight material (epicatechin and tannin dimers). The aqueous phase was retained and mixed with Amberlite FPX62 resin (500 mg/mL) using an orbital mixer (250 rpm; 8 h). The sample was filtered under vacuum through a glass microfiber filter to remove the resin and the filtrate was then loaded on a glass column packed with Amberlite XAD7HP (20–60 mesh, 500 cm^3) preconditioned with water (2 L). The column was washed with water (10 L) to remove any remaining anthocyanin, sugars and organic acids, then eluted with 80% aqueous ethanol (2 L). The eluate was rotary evaporated under reduced pressure (40 °C) to near dryness and further dried under vacuum with phosphorous pentoxide to yield a red-purple crusty solid (8.1 g).

2.3. Preparative HPLC fractionation of cacao tannin

The fractionation of cacao tannin by degree of polymerisation was achieved on an Agilent 1100 preparative high performance liquid chromatography (HPLC) system using a Develosil diol column (300 × 50 mm i.d. 10 μm) in the hydrophilic interaction liquid chromatography (HILIC) mode. The binary mobile phase consisted of (A) acetonitrile and (B) methanol/water/formic acid (99:0.95:0.05, v/v/v). Gradient conditions for separation and collection were 0–30% solvent B from 0 to 35 min, 30% isocratic solvent B from 35 to 65 min, 30–80% solvent B from 65 to 67 min, and 80% isocratic solvent B from 67 to 80 min. Prior to injection the column was allowed to equilibrate (0% solvent B, 10 min). Cacao tannin (1 g) was dissolved in a 6 mL mixture of 25:75 mobile phase A/B, centrifuged (16,100*g*, 10 min), and filtered through a 0.45 μm PTFE filter prior to injection (5000 μL). The flow rate was set at 55 mL/min and the column temperature was maintained at room temperature (~22 °C). Elution of separated cacao tannins was monitored by diode array detection (DAD) at 280 nm. Separated peaks were collected as they eluted, dried by rotary evaporation under reduced pressure (40 °C) then freeze dried to remove residual water and formic acid. Cacao fractions were prepared by this method three times for analysis.

2.4. HPLC-ESI-MS analysis of separated cacao tannins

The qualitative analyses of separated cacao tannins was performed by HPLC (Agilent 1100) interfaced with an ion trap mass spectrometer (G2445D Bruker, Billerica, MA, USA). HPLC analysis was performed on a 250 × 4.6 mm i.d. 5 μm Develosil diol column fitted with a guard column of the same phase. (Phenomenex, Torrance, CA). Cacao fractions were prepared by sonicating (10 min) cacao tannin (10 mg) in a mixture of the mobile phase (1 mL) containing solvent A/B (75:25, v/v). Prior to HPLC injection the sample was centrifuged (16,100 g, 5 min) then filtered through a 0.45 μm PTFE filter. The binary mobile phase consisted of (A) acetonitrile/acetic acid (98:2, v/v/v) and (B) methanol/water/acetic acid (95:3:2, v/v/v). HILIC separations were effected by a gradient of 0–40% solvent B from 0 to 60 min, 40–100% solvent B from 63 to 70 min, 100% isocratic B, and from 70 to 80 min, 100-0% B. Analysis was conducted with a 1.0 mL/min flow rate and a column temperature of 30 °C. Elution was monitored by DAD at 280 nm.

For mass spectral analysis, 10 mM ammonium formate was added to the eluent post column via a tee (50 μL/min) to enhance ionisation efficiency. Ionisation was achieved by electrospray ionisation in the negative mode with a nebulizer at 50 psi, drying gas at 10 L/min and drying temperature at 350 °C. The ionisation parameters were segmented during the HPLC separation to optimise ionisation of cacao tannins with increasing molecular mass. The segmented ionisation parameters are described in Table 1.

2.5. Protein precipitation of cacao tannins

2.5.1. Increasing tannin size

A 2 mg/mL stock solution of each cacao fraction was re-suspended in water. Aliquots from the stock solutions were then used to prepare tannin solutions in water of increasing concentration for each cacao fraction (50–2000 μg/mL). The iron reactive tannin content of each solution was determined against a standard curve prepared by reacting each corresponding tannin fraction with ferric chloride. The amount of iron reactive tannin precipitated in each solution by bovine serum albumin (BSA) protein was determined according to Harbertson, Kennedy, and Adams (2002) in triplicate. BSA has been used as model protein that mimics precipitation of salivary proteins with tannins that are connected to the sensation of astringency (Gawel, 1998; Green, 1993). Spectral analysis was conducted on a SpectraMax 384 UV–Vis absorbance microplate reader (Molecular Devices, Australia) using a polystyrene 96 well microplate (Greiner Bio-One, Interpath Services Pty. Ltd., West Heidelberg, Australia).