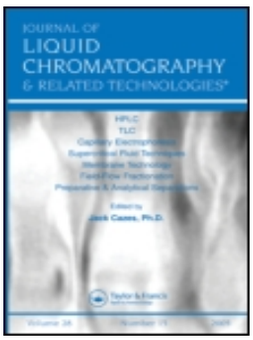

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Isolation of a Tetrameric A-type Proanthocyanidin Containing Fraction from Fresh Tea (*Camellia Sinensis*) Leaves Using High-Speed Counter-Current Chromatography

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A proanthocyanidin (PA) fraction containing tetrameric A- and B-type PAs was obtained after Sephadex LH-20 chromatography and high-speed counter-current chromatography (HSCCC) of a PA extract from fresh tea (Camellia sinensis) leaves. The biphasic solvent system hexane–ethyl acetate–methanol–water system (1:5:1:5), with the aqueous phase as the mobile phase, was selected on the basis of partition coefficient (K_D1.02), settling time (18 s), and stationary phase retention (60%). Electrospray ionization mass spectrometry (ESI-MS) indicated the presence of A-type linkages in the tetrameric PAs in one of the HSCCC fractions.

Keywords: A-type linkages, HSCCC, tea (*Camellia sinensis*), Sephadex LH-20, ESI-MS, tetrameric proanthocyanidins

Introduction

The wide distribution of oligomeric and polymeric proanthocyanidins (PAs) in the plant kingdom, their potent antioxidant properties and occurrence as active compounds in many plants and plant-derived extracts used in traditional medicines, and their ability to interact with biological systems have led to the isolation and study of PAs from different plants and plant parts.[1] Isolation and separation of proanthocyandins (PAs) from natural sources require a combination of elaborate chromatographic techniques, often involving HPLC, and are time consuming.

PAs are dimeric or oligomeric flavan-3-ols in which the monomeric units are linked by C–C bonds and have the typical C6–C3–C6 skeleton. Flavan-3-ols found commonly in PAs are (+)-afzelechin, (+)-catechin, and (+)-gallocatechin and their diastereomers (−)-epiafzelechin, (−)-epicatechin, and (−)-epigallocatechin. PAs made up of (epi)catechin are known as procyanidins, while propelargonidins and prodelphinidins contain at least one unit of (epi)afzelechin and (epi)gallocatechin, respectively. In B-type PAs interflavanol links are C–C bonds between C4 of one flavanol unit (upper unit) and the C8 or C6 of another (lower unit), Figure 1 (i). A-type PAs have two linkages (C–O and C–C) between two of the flavanol units, Figure 1 (ii).

A range of polyphenolic compounds, including flavan-3-ols (catechins), flavanol glycosides, and PAs, have been isolated from fresh tea leaves. PAs isolated from tea leaves, *Camellia sinensis* (L.) Kuntze var. *assamica*, comprise about 2% of the dry weight of the leaf. PAs from tea have been extracted into aqueous 70–80% acetone[2,3] and then separated using polyamide column chromatography and repeated Sephadex LH-20 column chromatography followed by HPLC. High-porosity polystyrene gel chromatography, followed by repeated Sephadex LH-20 chromatography combined with semipreparative HPLC have also been used to isolate dimeric and oligomeric PAs from green tea.[3–5]

Twenty one PAs, including a dimeric A-type PA, prodelphinidin A-2,3′-*O*-galllate, were isolated from an aqueous 80% acetone extract of Oolong tea after repeated chromatography over Sepahdex LH-20 and other reverse phase gels.[2] However, the methods used are time consuming and may cause irreversible adsorption of analytes on to the column material. To our knowledge this has been the only previous report of an A-type PA from tea.

High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid chromatography, has led to the efficient separation and preparative isolation of many classes of polyphenolics including catechins and PAs from green tea leaves.[6–8] Thin layer chromatography (TLC) combined with the use of *p-N,N*-dimethylaminocinnamldehyde (DMACA)[9] and different polar solvent systems has been used effectively to locate catechins and oligomeric proanthocyanidins on silica gel TLC plates.[10]

The present study describes the separation of a tetrameric PA fraction from an aqueous 70% acetone extract of fresh tea leaves using Sephadex LH-20 chromatography and HSCCC. Chromatography on Sephadex LH-20 using a limited volume of methanol enabled the separation of fractions that were rich in oligomeric PAs. TLC was used to analyze the fractions collected and

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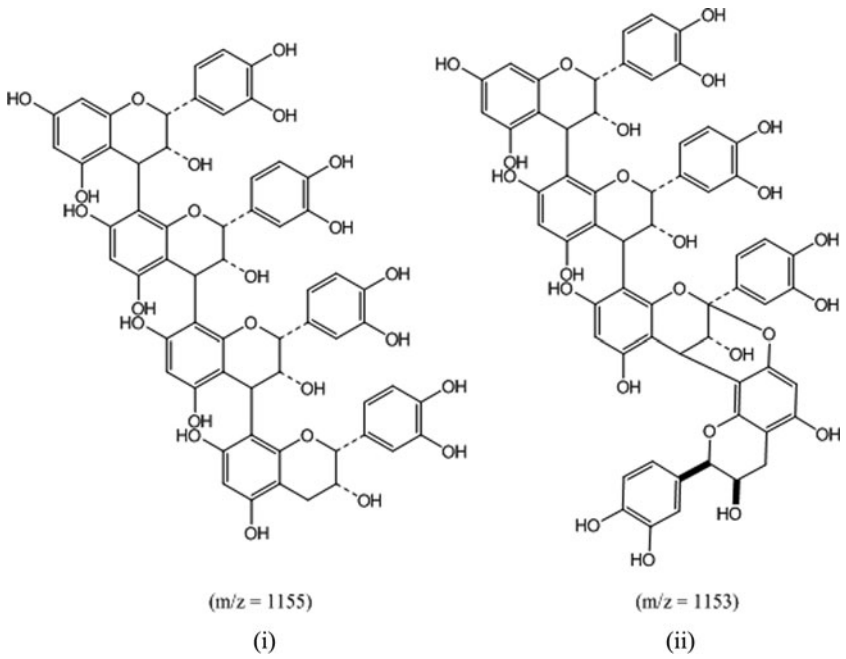

Fig. 1. Procyanidin structures: (i) B-type procyanidin tetramer and (ii) A-type procyanidin tetramer.

HSCCC was carried out only for the fractions containing polymeric PAs. Low-resolution electrospray ionization–mass spectrometry (LR-ESI-MS) of one of the PA fractions separated (PA_1) indicated that the fraction was a mixture of tetrameric PAs which contained both A-type and B-type PAs. Tetrameric A-type PAs from tea have not been separated before.

Experimental

Materials and Reagents

Tender tea shoots, also known as tea flush, were collected from cultivar TRI 2025 at the Tea Research Institute (TRI), Talawakelle, Sri Lanka. All fractions were analyzed using commercial TLC plates (Merck Silica Gel 60 F_{254}) and developed with the solvent system ethyl acetate–water–formic acid (90:05:05). The spots were visualized with DMACA spray reagent,[10] a 1% (w/v) solution of DMACA in methanolic sulfuric acid (8 mL H_2SO_4 and 100 mL methanol). PA containing fractions were identified by TLC as purplish blue spots with this reagent.

Extraction and Separation

PAs were extracted from freeze-dried tea leaves (100 g) into cold aqueous 70% acetone (200 mL) containing sodium ascorbate (0.2 g). The homogenate was filtered and saturated with sodium chloride (30 g) to facilitate the separation of two layers. The green upper phase containing PAs was collected and acetone was evaporated until chlorophyll precipitated. The filtered extract was partitioned with hexane (100 mL × 2) to remove lipids, concentrated, and freeze-dried to yield the PA extract as a pale brown powder (2.5 g). TLC indicated that the crude acetone extract from tea leaves was rich in PAs but contaminated with monomeric flavan-3-ol.

Purification by Sephadex LH-20 Chromatography

Preliminary purification was carried out on a Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) (20 × 2.3 cm) column. An aliquot of the PA extract (1.5 g in 9 mL of 50% aqueous methanol) was chromatographed on the LH-20 column (20 × 2.3 cm) pre-equilibrated with aqueous 50% methanol (800 mL) and then eluted using a limited volume of aqueous 50% methanol (270 mL) in order to remove catechins and separate PA rich fractions. Eluted fractions were monitored by TLC (solvent system) ethyl acetate–water–formic acid (90:05:05) (DMACA reagent)[10] and combined to give discrete fractions F_1 and F_2 until TLC indicated that PAs were being eluted. PA fractions were then eluted using aqueous 70% acetone and the eluted fractions were combined to give the discrete fractions F_3 and F_4. TLC indicated that fraction F_1(690 mg) contained only monomeric flavan-3-ols while fraction F_2(24 mg) was rich in monomeric flavan-3-ols but also contained PAs. Fractions F_3(83 mg) and F_4(237 mg), eluted with aqueous 70% acetone (100 mL), contained only PAs. Fraction F_4, obtained in the highest yield, was subjected to further separation by HSCCC.

HSCCC Procedure

All solvents used for HSCCC separation were distilled and degassed for 30 min to avoid accumulation of air bubbles inside the column and a Model CCC-1000-Pharma-Tech Research Corporation, Baltimore, MD USA, was used for the separation. The preparative coil (volume capacity of 300 mL) had an internal diameter of 2.6 mm, a β-value of 0.5–0.8, and a revolution radius of 7.5 cm.

Five two phase solvent systems containing different proportions of hexane–ethyl acetate–methanol–water (**A**, 1.5:1.0:1.5:1.0; **B**, 1:4:1:4; **C**, 1:5:1:5; **D**, 1:6:1:6; **E**, 1:7:1:7) were prepared and the partition coefficient (K_D) of each was determined

as described previously.[8] The hexane–ethyl acetate–methanol–water system **A** (1:5:1:5), with the aqueous phase as the mobile phase, was selected on the basis of partition coefficient (K_D 1.02). The settling time[11] and the stationary phase retention of this solvents system were also determined as described.[8,12]

A solution of the F_4 extract (200 mg in 5 mL of mobile phase) was injected into the HSCCC adjusted to head to tail mode with a revolution speed of 800 rpm. The fractions (~0.5 mL) were collected from the outlet of the column at an elution speed of 2.0 mL min^{-1} according to the absorbance plot recorded. The effluent from the outlet of the column was monitored continuously with a UV detector at 254 nm and collected manually (Figure 2). These fractions were analyzed by TLC and combined to give discrete fractions PA_1, PA_2, PA_3, PA_4, and PA_5. This process was repeated three times in order to get sufficient amounts of fractions PA_1–PA_5.

ESI-MS Spectrometry

Low-resolution ESI-MS spectra were run on a Thermo Electron Finnigan LTQ LC-MS system (Thermoscientific, FL, USA) with ESI system (ionization spray voltage 4.31 V, capillary voltage 2.97 V) at the Harbour Branch Oceanographic Institute at Florida Atlantic University. All compounds were dissolved in MeOH–water (1:1). A trace of formic acid was added for positive ion detection while a trace of NH_4OH was added for negative mode detection. The spectra were run by direct injection and the detector temperature was maintained at 225°C.

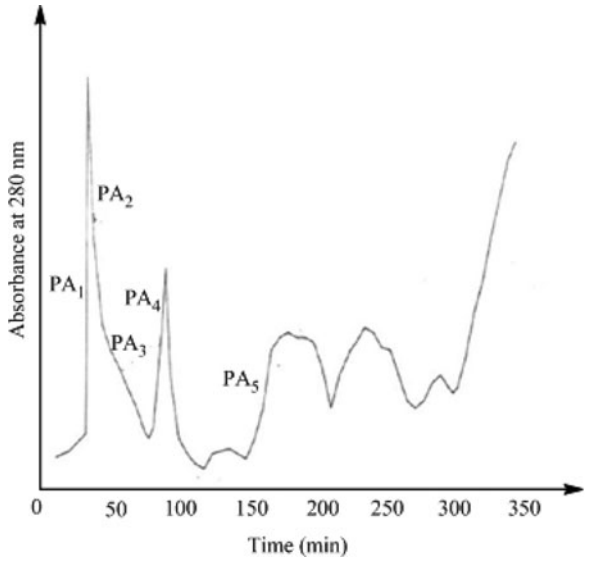

Fig. 2. HSCCC chromatogram for F_4 fraction and separation of fractions PA_1, PA_2, PA_3, PA_4, and PA_5. Experimental: Model CCC-1000-Pharma-Tech Research Corporation, Baltimore, MD USA: preparative coil (volume capacity of 300 mL) had an internal diameter of 2.6 mm, a β-value of 0.5–0.8, and a revolution radius of 7.5 cm; sample 200 mg; solvent system: hexane–ethyl acetate–methanol–water system (1:5:1:5); mobile phase: lower aqueous phase; head to tail elution; flow rate: 2.0 mL min^{-1}: revolution speed: 800 rpm; detection: 280 nm.

Results and Discussion

Separation of PAs Using Sephadex LH-20 Chromatography

The lipid free aqueous 70% acetone extract of freeze-dried tea leaves was found to contain catechins and PAs. Sephadex LH-20 chromatography was used to remove catechins and retain PA oligomers. Each eluted fraction (0.5 mL) was collected manually and monitored by TLC. Fractions were collected and combined to give the discrete fractions F_1–F_4 only after TLC indicated that PAs were being eluted. The PA extract was chromatographed on the Sephadex LH-20 column pre-equilibrated with aqueous 50% methanol (800 mL) and eluted using a limited volume of aqueous 50% methanol (270 mL) in order to remove catechins and separate PA rich fractions. Of these fractions F_1 and F_2 contained PAs but were rich in monomeric flavan-3-ols. Fractions F_3 and F_4 were rich in PAs and fraction F_4 obtained in the higher yield was subjected to HSCCC separation. Improved yields of PA rich fractions F_1–F_4 were obtained when a known weight of the freeze-dried crude acetone extract, rather than a concentrated crude acetone extract, was applied to the Sephadex LH-20 column.

Separation of PA Fractions by HSCCC

F_4 was subjected to further fractionation by HSCCC. Previous studies[8] had established that the two phase solvent system hexane–ethyl acetate–methanol–water (1:5:1:5) gave the best separations and in this study too, the same solvent system was selected for the separation on the basis of partition coefficient (K_D 1.02), settling time (18 s), and stationary phase retention (60%). Fractions were analyzed by TLC (DMACA reagent) and PA containing fractions PA_1 (52 mg), PA_2 (41 mg), PA_3 (41 mg), and PA_4 (26 mg) and PA_5 were obtained after three separate HSCCC runs of F_4(200 mg each) were performed (Figure 2). PA_5 shown on the chromatogram (Figure 2) was not collected and subjected to further study because TLC of the preliminary fractions of PA_5 eluted indicated that this fraction was rich in monomeric flavan-3-ols.

Four spots were observed on TLC of $PA_1$with R_fs of 0.26, 0.35, 0.6, and 0.76, respectively, and suggested that tetramers (R_f 0.26) as well as monomeric catechins and other PA oligomers were present in PA_1.[10] PA_1, separated in the highest yield by HSCCC, was studied by ESI-MS because TLC showed the presence of tetrameric PAs in this fraction. ESI-MS of fractions PA_2–PA_4 indicated that these three fractions were mixtures of tetrameric, trimeric, and dimeric PAs which we were unable to interpret unambiguously and are not reported in this paper

The molecular mass [M] obtained from low-resolution ESI-MS recorded for fraction PA_1 provided information regarding the degree of polymerization, the nature of the flavanol units, and presence of singly and doubly linked interflavan linkages in tetramers present in the PA_1 fraction. In the following discussion the notation (epi)catechin has been used, since the stereoisomers of catechin and epicatechin could not be distinguished during the ESI-MS analysis.

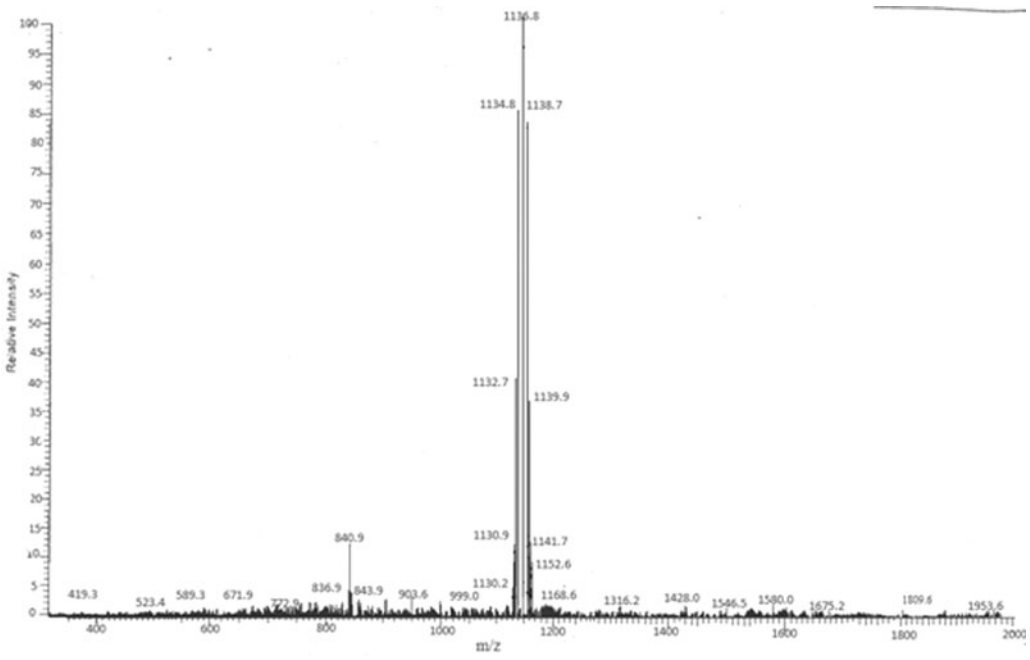

Fig. 3. ESI-MS of PA_1 (positive ionization mode). Thermo Electron Finnigan LTQ LC-MS system (Thermoscientific, FL, USA) with ESI system; ionization spray voltage: 4.31 V; capillary voltage: 2.97 V; sample: in MeOH–water (1:1) with a trace of formic acid; spectra run by direct injection; detector temperature: 225°C.

ESI-MS Mass Spectrometry

The ESI-MS spectrum (+ion) of PA_1 showed a cluster of peaks at m/z 1168.6, 1152.8, 1141.7, 1139.9, 1138.7, 1136.8 (base peak), 1134.8, 1132.7, and 1130.9 (Figure 3). The broad peaks in the M^+ ions region indicated structural heterogeneity in this fraction and were attributed to PA tetramers of varying composition in which some A-type linkages were present. A-type linkages are indicated when the M^+ ion is two mass units lower than expected.

The peak at m/z 1168.6 was consistent with an $(M+H)^+$ of a prodelphinidin tetramer composed of three (epi)catechin units, one (epi)gallocatechin unit, and with one A-type linkage, while the peaks at m/z 1152.8 to $(M+H)^+$ suggested a procyanidin tetramer with one A-type linkage, and the peak at m/z 1139.9 to $(M+H)^+$ of a B-type propelargodinin tetramer with three (epi)catechin residues and one (epi)afzelechin residue, respectively (Table 1).

The peaks with the highest signal height were those at m/z 1138.7, 1136.8, 1134.8, and 1132.7, and were attributed to the most abundant oligomers present in the PA_1 fraction. These peaks were assigned to PA tetramers with varying numbers of (epi)gallocatechin and (epi)afzelechin units and varying numbers of A-type linkages. The peak at m/z 1138.7 $(M)^+$ was attributed to a tetramer with three (epi)catechin residues and one (epi)afzelechin residue; m/z 1136.8 $(M)^+$ to a tetramer with three (epi)catechin and one(epi)afzelechin residues involving one A-type linkage (Table 1); m/z 1134.8 to a tetramer with three (epi)catechin units, one (epi)afzelechin unit, and two A-type linkages. It has been observed that PA oligomers of the cranberry flavanoid fraction which eluted with aqueous acetone from Sephadex LH-20 contained more A-type interflavan bonds.[13] The PA fraction under investigation was obtained from fraction F_4, which had been eluted from Sephadex LH-20 using acetone.

Table 1. (ESI-MS) Data[a] of PA_1 Fraction Separated from Fraction F_4 After HSCCC (Positive Ionization Mode)

Fraction	m/z	Assignments
PA_1	1168.6 $(M+H)^+$	Tetramer, one A-type linkage, three (epi)catechin residues, and one (ep)gallocatechin residue
	1152.8 $(M+H)^+$	Tetramer, procyanidin with one A-type linkage
	1141.7 $(M+H)^+$	Tetramer, B-type with three (epi)catechin residues and one (epi)afzelechin residue
	1139.9 $(M+H)^+$	Tetramer, three (epi)catechin residues and one (epi)afzelechin residue, one A-type linkage
	1138.7 $(M)^+$	Tetramer, three (epi)catechin residues and one (epi)afzelechin residue, one A-type linkage
	1136.8 $(M)^+$	Tetramer, three (epi)catechinresidues and one (epi)afzelechin residue, two A-type linkages
	1134.8, 1132.7, 1130.9	

[a]Low-resolution ESI-MS spectra run on a Thermo Electron Finnigan LTQ LC-MS system (Thermoscientific, FL, USA) with ESI system (ionization spray voltage 4.31 V, capillary voltage 2.97 V). Compounds were dissolved in MeOH–water (1:1). A trace of formic acid was added for positive ion detection while a trace of NH_4OH was added for negative mode detection. The spectra were run by direct injection; detector temperature maintained at 225°C.

The occurrence of tetramers with A-type PA residues in tea has not been reported previously. Hashimoto et al.[2] characterized a dimeric PA, prodelphinidin A-2,3′-*O*-gallate with $(M+H)^+$ 761. This is the only previous report that the authors have found for the occurrence of A-type PAs in tea. The presence of A-type PAs in tea is of considerable interest because A-type PAs have been implicated in the traditional use of cranberry juice for the treatment and prevention of urinary tract infections.[14]

Five dimeric PAs were isolated from tea by us in a previous study.[8] Four of these were prodelphinidins while one was a procyanidin. PAs with a higher degree of polymerization were not isolated. During the previous study,[8] the Sephadex LH-20 column was washed with a larger volume of aqueous 50% MeOH (2.5 l) until TLC confirmed that non-PA components such as catechins were not being eluted. The separation of tetrameric PAs during the present study was made possible by limiting the amount of aqueous 50% methanol (270 mL) that was used for elution of low molecular weight carbohydrates and catechins from the Sephadex LH-20 column. Limiting the volume of aqueous 50% methanol used for elution prevented over washing of oligomeric PAs.

Conclusions

The isolation of tetrameric A-type PAs from an aqueous acetone extract of fresh tea leaf was achieved by the combined use of Sephadex LH-20 chromatography and HSCCC. Separation of tetrameric PA containing fractions was made possible by eluting the pre-equilibrated Sephadex LH-20 column with a limited volume of 50% aqueous methanol.

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