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Isolation and identification of oligomeric procyanidins from *Crataegus* leaves and flowers

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Abstract

Oligomeric procyanidins were isolated from the leaves and flowers of hawthorn (*Crataegus laevigata*). A trimer, epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin, and a pentamer consisting of (−)-epicatechin units linked through C-4β/C-8 bonds have been isolated from hawthorn for the first time, in addition to known procyanidins including dimers B-2, B-4 and B-5, trimers C-1 and epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin, and tetramer D-1. A fraction containing a hexamer was also found.

Keywords: *Crataegus laevigata*; Rosaceae; Hawthorn; Oligomeric procyanidins; Condensed tannins; ESI–MS

1. Introduction

Hawthorn (*Crataegus* sp.) is a traditional European medicinal plant. The species most often used are *Crataegus monogyna* and *Crataegus laevigata* (ESCOP, 1992). Dried flowers, leaves and fruits are used as crude drugs. Several studies have shown that aqueous and alcoholic hawthorn extracts have beneficial effects on the heart and blood circulation (Ammon and Kaul, 1994a,b). Oligomeric procyanidins and (−)-epicatechin are considered to be the main active constituents, in addition to flavone- and flavonol-type flavonoids (ESCOP, 1992).

Procyanidins are a class of proanthocyanidins (condensed tannins) consisting of flavan-3-ol units, epicatechins and/or catechins. Flavanol units are primarily interlinked through C-4/C-8 linkages, but a C-4/C-6 and a double interflavanoid linkage (C–C and C–O) may also exist. Procyanidins can be categorized as oligomeric procyanidins (OPs) consisting of 2–6 flavanol units, and polymeric procyanidins (PPs) consisting of more than six flavanol units (Haslam, 1998). Dimeric procyanidins and a trimer, C-1, have previously been isolated from *C. monogyna* (Thompson et al., 1972). Rohr (1999) isolated the procyanidins B-2, B-4, B-5, C-1, epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin, and a tetramer, D-1, from *Crataegus* leaves and flowers. However, the isolation of larger procyanidins has not been reported, and the procyanidin content in *Crataegus* sp. is largely unknown. Quality control and standardization of hawthorn preparations and plant material have been found to be inadequate with respect to oligomeric procyanidins. A prerequisite for developing such a standard method is the isolation of individual oligomeric procyanidins because commercial reference substances are not available. Instability and diversity of procyanidins especially complicate the isolation of individual compounds. Procyanidins have been separated into distinct oligomeric classes on the basis of their degree of polymerization by means of normal-phase HPLC (Yanagida et al., 2000), and by high-speed counter-current chromatography (Shibusawa et al., 2001).

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In recent years, attention has been paid to polyphenolic procyanidins as a result of their antioxidant and radical scavenging activities, but little is currently known about their pharmacokinetics in human (Saint-Cricq de Gaulejac et al., 1999). Although procyanidins are widely distributed in nature (Haslam, 1998), their structures and concentrations in plants and commonly consumed foods are not known. Therefore, suitable methods for isolating and determining procyanidins are needed. The purpose of this study was to isolate oligomeric procyanidins from flowers and leaves of hawthorn by column chromatography, and to determine the structures of the isolated compounds.

2. Results and discussion

A series of oligomeric procyanidins from monomer **1** to hexamer **10** was isolated from the leaves and flowers of hawthorn by open-column liquid chromatography (CC), using polyamide and Sephadex-LH 20 as stationary phases by modifying the method of Thompson et al. (1972). In this work, elution of the polyamide column was also performed with mixtures of methanol-water and acetone-water, resulting in successful elution of tetrameric **8**, pentameric **9** and hexameric **10** procyanidins. These chromatographic methods can be easily adapted to large-scale isolation.

Compound **1**, (−)-epicatechin, was identified using the reference compound. Compound **2** was the main dimer, epicatechin-(4β→8)-epicatechin (procyanidin B-2), **3** catechin-(4α→8)-epicatechin (procyanidin B-4), and **4** epicatechin-(4β→6)-epicatechin (procyanidin B-5).

Compound **5** was the main trimer, epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (procyanidin C-1), compound **7** epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin, and **8** the main tetramer, epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin(4β→8)-epicatechin (procyanidin D-1). The same procyanidins have previously been reported from *Crataegus* leaves and flowers by Rohr (1999). Compounds **6** and **9** were both isolated from hawthorn for the first time.

The negative ESI–MS of 6 showed a signal at m/z 865 corresponding to a deprotonated trimer. The linkage 4β→8 between flavanol units I and II of **6** was determined on the basis of procyanidin B-2 4′-benzylthioether, and the linkage between flavanol units II and III was concluded on the basis of a free dimer, procyanidin B-5, released in partial acid hydrolysis. Accordingly, compound **6** was identified as epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin.

The positive ESI–MS of **9** indicated a pentaflavanoid structure (M 1442) as well as a fragment ion at m/z 865 corresponding to a carbocationic trimer, and at m/z 579 to a protonated dimer. The flavanol units II–V of **9** were deduced from partial acid hydrolysis which gave, in addition to a free (−)-epicatechin, dimer B-2, trimer C-1 and tetramer D-1 that all consisted of (−)-epicatechin units linked through 4β→8 bonds. In addition, **9** was decomposed by complete thiolysis into its monomeric units, and only (−)-epicatechin and a epicatechin-benzylthioether derivative were obtained, confirming that **9** consisted entirely of (−)-epicatechin units. The linkage 4β→8 between the flavanol units I and II was concluded on the basis of a B-2 4′-benzylthioether. Consequently, the structure of **9** was deduced to be epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (procyanidin E-1).

The positive ESI-MS of **10** exhibiting signals at m/z 1731 $[M+H]^+$ and at m/z 1769 $[M+K]^+$ showed a hexaflavanoid constitution (M 1730) as well as fragment ions at m/z 579, 867, 1155 and 1443 corresponding to a protonated dimer, trimer, tetramer and pentamer, respectively.

The structures of the isolated compounds were elucidated on the basis of their UV and mass spectra, and through acid-catalysed decomposition. The isolated OPs had identical absorption maxima in their UV absorption spectra: 234 and 279 nm. The molecular weights were determined primarily by electrospray-ionization–mass spectrometry (ESI–MS) in the positive ion mode. Protonation is possible due to the sufficiently high proton affinity of procyanidins. Being phenols, procyanidins are also sufficiently acidic for ionization in solution by deprotonation. In addition to the protonated molecule, sodium and potassium adduct ions and double-charged ions provided molecular weight information. Fragmentation of procyanidins occurred, and ions corresponding to the subunits of procyanidins were found in the mass spectra. The mass spectral analyses of compounds **6** and **7** were performed using ESI in the negative ion mode.

All the isolated compounds were verified by complete acid hydrolysis as procyanidins, yielding a red cyanidin. Flavanol units and interflavanoid bonds were determined by hydrolytic cleavage. The compounds were decomposed into monomeric and oligomeric subunits by partial acid hydrolysis. The upper flavanol unit (I) of a procyanidin breaks away as a carbocation, and the lower one (II) exists as a free compound (Thompson et al., 1972) (Fig. 1).

Acid-catalysed degradation was also performed in the presence of benzyl mercaptan or phloroglucinol. A nucleophilic benzyl mercaptan reacts with a carbocation forming one thioether derivative with (−)-epicatechin (t_R 36.7 min), and two derivatives with (+)-catechin (t_R 35.3 min and 36.4 min). Thioderivatives were desulphurized, and the flavanol unit I was identified on the basis of the formed monomer. Two upper flavanol units (I and II) and their interflavanoid linkage in larger procyanidins were identified on the basis of a thioether derivative formed from the released dimer. Phloro-

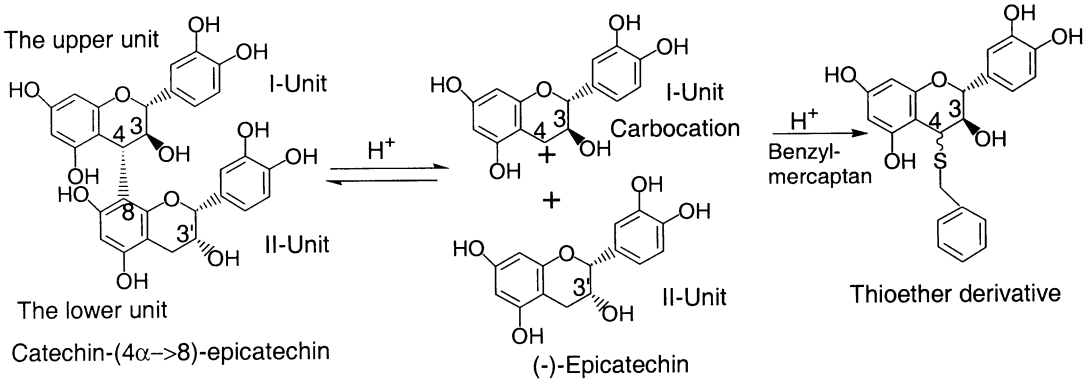

Fig. 1. Partial acid hydrolysis of a procyanidin dimer in the presence of benzyl mercaptan.

glucinol reacts with the carbocation forming with (−)-epicatechin one derivative and with (+)-catechin two derivatives. Identification of degradation products was performed by chromatography on TLC and HPLC starting with the identification of three dimeric procyanidins. According to literature, stereochemistry of the interflavanoid bonds is always *trans* to the C-3 hydroxyl group (Haslam, 1998). Fletcher et al. (1977) have proved by NMR spectra that the orientation of the interflavan bond is β at C-4 in the upper flavan-3-ol unit I if it is (−)-epicatechin (3*R*). If the upper flavan-3-ol unit is (+)-catechin (3*S*), the orientation of the interflavan bond at C-4 is α. This kind of α,β orientation of the interflavanoid bonds in larger procyanidins has also been revealed by NMR spectra (Rohr, 1999; Morimoto et al., 1986), and the structures of OPs have been established by means of chemical degradations (Morimoto et al., 1986).

3. Experimental

3.1. Materials

Flowers and leaves of *Crataegus laevigata* (*C. oxyacantha*) were kindly donated by Flachsmann AG, Germany. (+)-Catechin and (−)-epicatechin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and cyanidinchloride from Carl Roth, Germany.

3.2. Extraction and isolation of compounds *1–10*

The compounds were extracted from 50 g of milled leaves and flowers of hawthorn with methanol-water (7:3) (2×150 ml) and methanol (200 ml), the mixture being kept in an ultrasonic bath for 15 min. The combined extracts were evaporated to a smaller volume (200 ml) at room temperature (below 35 °C) and extracted with petroleumbenzin (3×200 ml). The remaining water–methanol extract was further extracted with ethyl acetate (3×200 ml). The ethyl acetate phase was evaporated to dryness, and the raw extract (2.5 g) was transferred to a polyamide CC 6 column (30 cm×25 mm i.d.) (column I). Elution was performed with methanol (700 ml), methanol–water (7:3) (600 ml) and acetone-water (7:3), and fractions (12 ml) were collected. The flow rate was 1.5 ml/min. Elution of the compounds was monitored by TLC and HPLC-DAD. The eluents were evaporated under reduced pressure (below 35 °C), and the residue was freeze-dried.

Compound **1** (R_f 0.80, t_R 18.6 min) was isolated in fractions 18–33 from column I, and purified by preparative HPTLC. The band was isolated from the plate, and **1** was extracted using methanol.

Compounds **2–4** were isolated in fractions 43–75 and 117–121 from column I. The combined fractions were rechromatographed on a Sephadex LH-20 column (33 cm×15 mm i.d.). Elution was performed using ethanol, and fractions (10 ml) were collected. Compound **2** (R_f 0.62, t_R 17.2 min) was isolated in fractions 20–28, 3 (R_f 0.61, t_R 15.6 min) in fractions 30–33, and **4** (R_f 0.71, t_R 23.9 min) in fractions 36–42. +ESI–MS data of **2–4** were (*m/z*): $[M+H]^+$ 579, $[M+Na]^+$ 601, $[M+H-290]^+$ 289, and a fragment at 291.

Compounds **5–7** were isolated in fractions 123–140 from column I. The combined fractions were rechromatographed with ethanol on a Sephadex LH-20 column (33 cm×15 mm i.d.) (column II), and fractions (10 ml) were collected. Compound **5** (R_f 0.45, t_R 19.5 min, $[M+H]^+$ at *m/z* 867) was isolated from fractions 36–46. Compound **6** (R_f 0.49, t_R 24.2 min, $[M–H]^-$ at *m/z* 865) was isolated from fractions 55–60, and compound **7** (R_f 0.46, t_R 16.9 min, $[M–H]^-$ at *m/z* 865) from fractions 61–67 by semi-preparative HPLC. Compound **6** formed one epicatechin-phloroglucinol derivative and **1**. Partial acid hydrolysis released from **6** compounds **1** and **4**. Partial thiolysis of **6** formed epicatechin-benzylthioether, procyanidin B-2 4′-benzylthioether, **1** and **4**.

Compound **8** (R_f 0.28, t_R 20.0 min, +ESI–MS m/z: $[M+H]^+$ 1155, $[M+K]^+$ 1193, $[M+K+H]^{2+}$ 597) was isolated in fractions 150–157 from column I.

Compound **9** (R_f 0.11, t_R 21.0 min) was isolated in fractions 179–183 from column I, +ESI–MS m/z: $[M+H]^+$ 1443, $[M+K]^+$ 1481, $[M+K+H]^{2+}$ 741, $[M+H\text{-}578(\text{dimer})]^+$ 865 and a fragment at 579. Partial acid hydrolysis released compounds **1**, **2**, **5** and **8**. Partial thiolysis formed epicatechin-benzylthioether, procyanidin B-2 4′-benzylthioether, **1** and **2**. The unit I formed one derivative with phloroglucinol and **1**. Complete thiolysis yielded **1** and epicatechin-benzylthioether.

Compound **10** (R_f 0.06, t_R 21.5 min) was isolated from column I in fractions 200–215 containing also a pentamer. +ESI–MS of **10** m/z: $[M+H]^+$ 1731, $[M+K]^+$ 1769, $[\text{hexamer}+K+H]^{2+}$ 885, $[\text{pentamer}+K+H]^{2+}$ 741, fragment ions at 579, 867, 1155 and 1443.

3.3. Chromatographic and spectrometric methods for isolation and identification

The TLC separations were performed with ethyl acetate/formic acid/acetic acid/water (75:3:2:20) according to Vanhaelen and Vanhaelen-Fastre (1989). The adsorbents were Kieselgel 60 F_{254} aluminium plates 20×20 cm, 0.25 mm (Merck), and Kieselgel 60 F_{254} HPTLC 10×10 cm (Merck). The spots were made visible with vanillin (1%)-sulphuric acid. The HPLC system consisted of a Waters 600E Multisolvent Delivery System, autosampler 717 and photodiode array detector 991 (Millipore, USA). The elution conditions were as described in Rigaud et al. (1991): solvent A 2.5% acetic acid, solvent B acetonitrile/2.5% acetic acid (80:20). Linear gradients: solvent B 5–50% in 35 min, 50–100% in 40 min. Flow rate 1 ml/min, detection at 280 nm. Column: LiChroCart, 250–4, Hypersil ODS (5 μm), Merck. The elution conditions in semi-prep. HPLC were: solvent A 2.5% acetic acid, solvent B methanol/2.5% acetic acid (80:20), solvent B 0–30% in 20 min.

Positive ion ESI mass spectra were recorded on a Micromass Quattro II triple quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) with an electrospray interface operating capillary voltage of 2.3 or 2.4 kV and a source temperature of 70 °C. The cone voltage varied from 32 to 36 V. Negative ion ESI mass spectra were recorded on a PE Sciex API 300 triple quadrupole system (Sciex, Toronto, Canada) with an ionspray interface. The ionspray and orifice voltage were maintained at −4 kV and −35 V, respectively. The samples were dissolved in acetonitrile–water (1:1) containing formic acid (0.1%). The flow rate was 5 μl/min.

3.4. Hydrolytic cleavage

Complete and partial acid hydrolyses were performed as described in Thompson et al. (1972). However, the reaction mixture containing tetramer **8** was heated for partial acid hydrolysis for 6 min, and that with pentamer **9** for 2.5 min only. Hydrolytic cleavage in the presence of phloroglucinol and benzyl mercaptan was performed acccording to Ricardo da Silva et al. (1991). Complete thiolysis was performed by modifying the method of Morimoto et al. (1986), the reaction mixture, consisting of 0.5 mg procyanidin, 0.2 ml benzyl mercaptan (5% in ethanol) and 50 μl acetic acid, was heated in a vial for 60 min at 95 °C. HPLC analyses of different hydrolytic cleavages were conducted under the same conditions, using elution conditions as described in Rigaud et al. (1991).

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