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Procyanidins from the Roots of *Fragaria vesca*: Characterization and Pharmacological Approach

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Water-soluble procyanidins obtained by fermentation of a tannin extract of roots of *Fragaria vesca* were analyzed by high performance liquid chromatography. Three dimers, procyanidins B_1, B_2 and B_5, and two monomers, (+) catechin and (−) epicatechin, were identified and quantified. These procyanidins exhibit antibacterial and marked angioprotective properties.

Keywords——*Fragaria vesca*; fermentation; *Saccharomyces rouxii*; procyanidin; HPLC; antibacterial activity; angioprotective activity

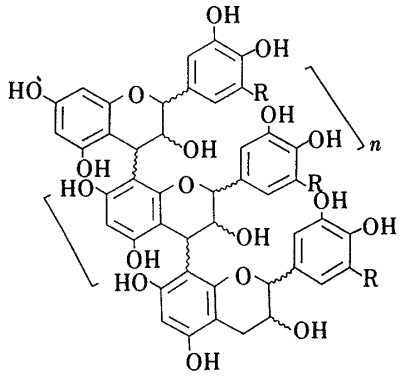
Tannins, and particularly proanthocyanidin polymers have attracted interest for nearly two centuries. The structures of proanthocyanidin condensates, which had long remained problematic, became accessible with the advent of techniques such as ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) [1-5] and mass spectrometry.[6]

In plants, proanthocyanidins always occur as a mixture of oligomers and polymers and so their structural analysis requires a prior fractionation stage usually involving Sephadex LH20 column chromatography[7] or high performance liquid chromatography (HPLC).[3,8] The characterization of the fractions thus obtained has been thoroughly described.[9-13] However, little research has been done on the preparation of water-soluble proanthocyanidins of homogeneous molecular weight in quantities sufficient for pharmacological testing. Attempts at obtaining such material have often failed due to the use of extraction and purification techniques ill-suited to the chemical nature of the polymers.

Fermentation methods had, in our hands, proved useful in circumstances where conventional chemistry was of no avail[14,15] and so we turned to such techniques for fractionating proanthocyanidin polymers extracted from rhizomes of *Fragaria vesca* (Rosaceae).

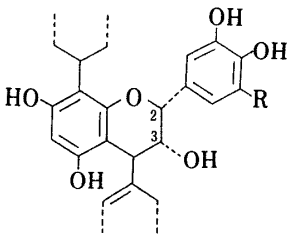
Depolymerization was carried out by using a strain of *Saccharomyces rouxii* at 27 °C in 30% ethanol (v/v). This medium dissolves a high proportion of the polymeric material and also ensures that the fermentation is free of microbial contamination.[16] Depolymerization, the extent of which is maximal after 108 h, results in a marked increase in the water-solubility of the tannin extract, which rises from 43% to up to 75%.[17] The water-soluble proanthocyanidins thus obtained were structurally analyzed[18] based on the criteria of Czochanska *et al.*[19]: the relative proportions of procyanidin (1) or PC (3) units and prodelphinidin (2) or PD (4) units, *i.e.* the ratio (3):(4), the stereochemistry of the heterocycle of the monomer units, *i.e.* the relative proportions of *cis* (5) and *trans* (6) units (ratio (5):(6)), and the degree of polymerization (number of monomer units *n*).

Chromatography on Sephadex LH 20 columns followed by ^{13}C-NMR analysis gave



1, 3 : R=H
2, 4 : R=OH

Chart 1



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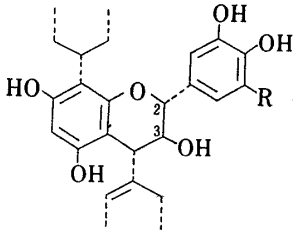

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Chart 2

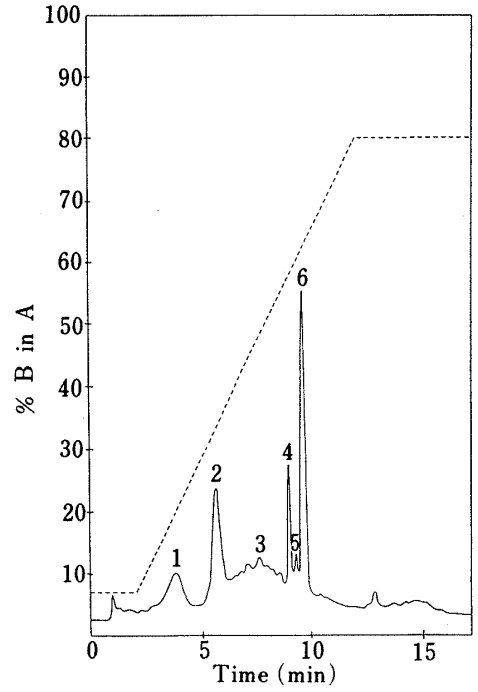


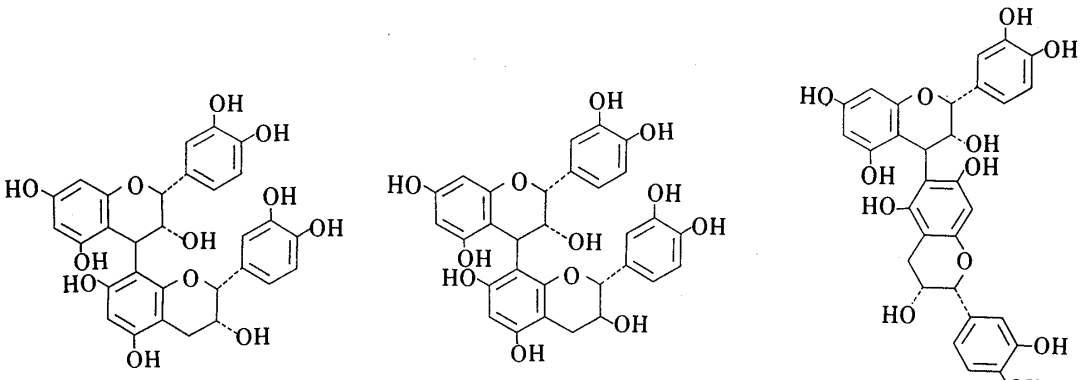
Fig. 1. HPLC of Procyanidins over Lichrosorb RP-18 10 μm

1, unknown; 2, (+)catechin; 3, (−)epicatechin; 4, procyanidin B_2; 5, procyanidin B_5; 6, procyanidin B_1.

(3) : (4) = 100 : 0, (5) : (6) = 75.5 : 24.5 and $n \leq 3$.[20]

In the first part of this paper we describe the analysis of these procyanidins by HPLC. Inspection of the published work on HPLC analysis of proanthocyanidins reveals that the best results have been obtained with reversed phase columns with a gradient of methanol in aqueous acid.[21,22] We developed a method of chromatography on Lichrosorb RP-18 10 μm with a gradient of methanol in 5% aqueous formic acid. The chromatogram obtained is shown in Fig. 1. Co-injection of controls allowed three dimers to be identified, procyanidins B_1 (7), B_2 (8) and B_5 (9), along with two monomers, (+)catechin (10) and (−)epicatechin (11).

Quantitative analysis of the mixture required improved resolution, which was achieved by using the same gradient on a column of Lichrosorb RP-18 5 μm instead of 10 μm. The chromatogram, shown in Fig. 2, has a much better resolution particularly for (+)catechin, (−)epicatechin and procyanidin B_5. The concentrations of the main components of the mixture were determined by integration of peak areas. As shown in Table I, the monomers and the three dimers B_1, B_2, B_5 account respectively for 26.9 and 47.3% of the chromatog-



7 8 9

Chart 3

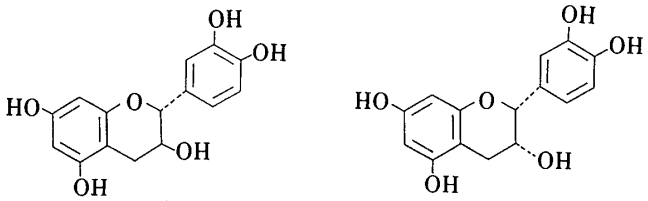


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Chart 4

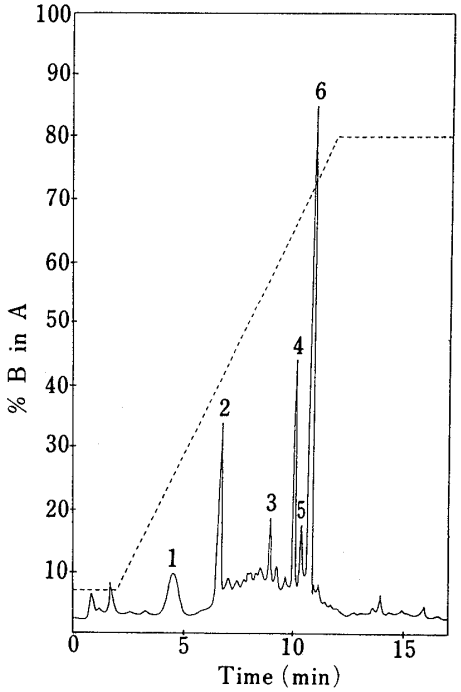

Fig. 2. HPLC of Procyanidins over Lichrosorb RP-18 5 μm

1, unknown; 2, (+)catechin; 3, (−)epicatechin; 4, procyanidin B_2; 5, procyanidin B_5; 6, procyanidin B_1.

TABLE I. HPLC Analysis of Procyanidins over Lichrosorb RP-18 5 μm

Procyanidin	t_R (min)	Concentration (%)
Unknown	4.60	15.0
(+)Catechin	6.60	22.3
(−)Epicatechin	8.95	4.6
Procyanidin B_2	10.00	16.4
Procyanidin B_5	10.25	1.6
Procyanidin B_1	10.60	29.3

raphed sample.

HPLC on Lichrosorb RP 18 5 μm thus allowed a full structural analysis of water-soluble procyanidins obtained by fermentation. These are made up essentially of the dimers B_1, B_2 and B_5 accompanied with monomers, (+)catechin and (−)epicatechin

Recent work on proanthocyanidin oligomers has pointed to potential pharmacological applications of B series procyanidin dimers.[23−25] However, the very small quantities of procyanidin B available have restricted the study of such applications. The fermentation process described above provided us with a homogeneous mixture of monomers and dimers in sufficient quantity for pharmacological testing.

Proanthocyanidins confer two properties on the plants containing them, namely an antibacterial activity and more importantly an angioprotective capability. Pourrat *et al.* have demonstrated the bacteriostatic and agglutinant activities of tannin extracts of rhizomes of

TABLE II. Bacteriostatic Activity of Tannic Extract of *Fragaria vesca*

Bacterium	Bacteriostatic activity		
	2.5 mg/ml	5 mg/ml	10 mg/ml
Escherichia coli 055 B5	−	−	±
Escherichia coli 0111 B4	−	−	±
Pasteurella pseudotuberculosis type 1	±	+	+
Proteus mirabilis	−	−	−
Proteus vulgaris	±	±	+
Pseudomonas aeruginosa	±	±	+
Shigella boydii	±	+	+
Shigella flexneri type 1	+	+	+
Shigella sonnei type 1	+	+	+
Staphylococcus aureus	±	±	+
Streptococcus faecalis	−	±	+

+, total inhibition of bacterial growth; ±, partial inhibition; −, absence of inhibition.

TABLE III. Angioprotective Activity of Procyanidins Obtained by Fermentation

Tested substance	Dose mg/kg i.p.	Number of experiments	OD_{623}		% activity/ group 1	Statistical analysis
			M	*Δ*m		
Group 1						
HPMC 0.03% in NaCl 9%		4	0.361	0.011	0	$p<0.001$
Group 2						
Anthocyanins of *Vaccinium myrtillus*	200	4	0.452	0.010	25.2	$p<0.001$
Group 3						
Procyanidins	50	7	0.392	0.006	8.6	$p<0.001$
	100	7	0.401	0.010	11.1	$p<0.001$
	200	7	0.437	0.014	21.1	$p<0.001$

HPMC, hydroxypropylmethylcellulose.

Fragaria vesca, in vitro.[26] These totally inhibited the growth of most of the bacteria tested, at 10 μg/ml (Table II), and in addition exhibited an agglutinating activity towards suspensions of *Salmonella*, *Shigella*, *Brucella* and *Pasteurella*. Extracts were also tested clinically on subjects with serious pyocyanic affections and were found to possess a bactericidal potency *per os* at doses of several grams per day.[27] Though this potency is weak, such treatment may be useful in cases of marked resistance to antibiotics. It is now known that the angioprotective effects ascribed to the proanthocyanidins in various tannin extracts are mainly due to the oligomers.

The angioprotective activity of the homogeneous mixture of monomers and dimers of procyanidins obtained by fermentation was investigated in the laboratory of Professor P. Bastide. A well-codified pharmacological test was applied, that of Beach and Steinetz[28] which consists of the evaluation in the rat of capillary permeability in terms of the diffusion of a macromolecular dye, Evans blue. The dye is administered to the rats intravenously. Fifteen minutes later, its plasma concentration is determined by spectrophotometric assay. Administration of an angioprotective agent delays the diffusion of the Evans blue out of the general circulation.

The anthocyanins of *Vaccinium myrtillus* were used as a reference. The results of the test are summarized in Table III. At 50 and 100 mg/kg, the procyanidins only slightly delayed the

elimination of the dye. However, at 200 mg/kg, they exhibited a marked angioprotective activity, amounting to about 84% of that of the reference anthocyanins at the same dose.

Experimental

Isolation of Procyanidins——*Fragaria vesca* roots were powdered and extracted with 80% (v/v) ethanol. The yield of this extraction was about 25%. Tanning extract was depolymerized by fermentation as described previously.[17] Water-soluble procyanidins thus obtained (100 mg) were dissolved in 2 ml of MeOH–H_2O (1 : 1) and applied to a column of Sephadex LH-20. Purified procyanidins were eluted as a discrete, visible band with 150 ml of EtOH. This solvent was removed *in vacuo* at 40 °C.

HPLC——Analyses were conducted with two model 510 pumps, a 680 solvent programmer, a U6K injector and a UV detector, model 490 (Water Associates Inc.). A Hitachi D 2000 integrator-calculator was used for all computations.

HPLC over Lichrosorb RP-18 10 μm (250 × 4 mm) (Merck) was carried out as follows. Gradient: two solvents were used (A) HCO_2H–H_2O (5 : 95) (B) MeOH. The elution profile was: 0—2 min, 7% B in A (isocratic); 2—12 min, 7—80% B in A (linear gradient); 12—17 min, 80% B in A (isocratic). Flowrate: 2.5 ml/min. Injection volume: 20 μl. Temperature: 20 °C. UV detection : 280 nm, 0.2 a.u.f.s. t_R (+) catechin = 6.00 min; t_R (−) epicatechin = 7.80 min; t_R procyanidin B_2 = 9.15 min; t_R procyanidin B_5 = 9.50 min; t_R procyanidin B_1 = 9.80 min.

Analysis over Lichrosorb RP-18 5 μm (125 × 4.6 mm) (Merck) was performed with the same gradient under the following conditions. Flow-rate: 1.7 ml/min. Injection volume: 10 μl. Temperature: 20 °C. UV detection: 280 nm, 0.2 a.u.f.s. t_R (+)catechin = 6.60 min; t_R (−)epicatechin = 8.95 min; t_R procyanidin B_2 = 10.00 min; t_R procyanidin B_5 = 10.25 min; t_R procyanidin B_1 = 10.60 min.

Angioprotective Analysis——Male Iffa Credo OFA rats weighing 180 to 200 g each were used. Animals were grouped into 3 groups and fasted for 12 h prior to the beginning of the experiment.

Group 1 received a 0.03% HPMC solution in 9% NaCl intraperitoneally (5 ml/kg body weight). Group 2 received intraperitoneally anthocyanins of *Vaccinium myrtillus* dissolved with HPMC solution, at 200 mg/kg body weight. Group 3 received intraperitoneally procyanidins dissolved in HPMC solution. Three doses were tested: 50, 100 and 200 mg/kg body weight.

One hour later, a 1% Evans blue solution in 9% NaCl was injected intravenously into the rats (2.5 ml/kg body weight).

At 15 min after this injection, a sample of blood was taken in the retroorbital sinus and spun for 2 × 2 min at 18000 rpm; 50 μl of the upper phase was diluted with 2.5 ml of 9% NaCl.

The color intensity of diluted plasma was estimated by spectrophotometry at 623 nm.

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