Cell Wall-Bound Phenolics from Norway Spruce *(Picea abies)* Needles

Dieter Strack*, Jürgen Heilemann, and Eva-Susan Klinkott

Botanisches Institut der Universität zu Köln, Gyrhofstraße 15,
D-5000 Köln 41, Bundesrepublik Deutschland

Victor Wray

Gesellschaft für Biotechnologische Forschung (GBF), Mascheroder Weg 1,
D-3300 Braunschweig, Bundesrepublik Deutschland

Z. Naturforsch. **43c,** 37–41 (1988); received August 25, 1987

Picea abies (L.) KARST., Hydroxycinnamic Acids, Flavonols, Cell Wall, Localization

Insoluble phenolics have been isolated and identified from Norway spruce (*Picea abies* [L.] KARST.) needles as cell wall-bound astragalin (kaempferol 3-O-β-glucoside) and *p*-coumaric acid as major components, and ferulic acid as a minor one. They probably mainly occur as lignin-carbohydrate complexes.

Introduction

Secondary products, such as phenolics, accumulate predominantly in the form of hydrophilic conjugates in the vacuoles (intraprotoplasmic storage) of plants [1–3]. Also there are many reports on their extraprotoplasmic location, *e.g.* as exudates [4] or cell wall components. The latter include the polymer lignin [5], as well as monomeric hydrolyzable phenolics, which are mainly hydroxycinnamic acids (HCAs; ferulic, diferulic, and *p*-coumaric acids) linked to polysaccharides [6–11] and/or lignin [12–14]. In contrast, the most widespread flavonoids have rarely been found to be associated with cell walls. Markham [15] isolated a flavone polysaccharide from *Monoclea forsteri* which was possibly cell wall bound, while Ibrahim *et al.* [16] showed, with the aid of immunological methods, that a methylated flavonol glycoside was probably located in the cell walls of *Chrysosplenium americanum*.

In the course of an investigation of the phenolics in Norway spruce (*Picea abies* [L.] KARST.) needles we observed both HCA- and flavonoid-like insoluble hydrolyzable compounds. We report here their identification and localization and present evidence for cell wall-bound astragalin (kaempferol 3-O-β-glucoside) and *p*-coumaric acid as major components, and ferulic acid as a minor one.

Abbreviations: HCA, hydroxycinnamic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

* Present address: Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig, Mendelssohnstraße 1, D-3300 Braunschweig.

Reprint requests to Prof. Dr. D. Strack.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0100–0037 $ 01.30/0

Materials and Methods

Plant material

Needles (2nd year) of 35- to 40-year-old *Picea abies* (L.) KARST. (syn. *P. excelsa* [LAM.] LINK) were collected from natural locations in Nordrhein-Westfalen (near Bad Münstereifel, FRG) and were immediately frozen with liquid nitrogen. Once in the laboratory, they were stored at −30 °C until they were processed as described below.

Chemicals and enzymes

p-Coumaric acid, ferulic acid, and nicotiflorin (kaempferol 3-O-rutinoside) were from Roth (Karlsruhe, FRG). Driselase came from Sigma (Deisenhofen, FRG) and was purified as described elsewhere [9]. "Hemicellulase" (Sigma) contained per mg protein 0.053 and 0.036 activity units of hemicellulase and cellulase, respectively. All other chemicals and solvents used were of analytical grade.

Cell wall preparation

Crude preparation. Norway spruce needles (1 g aliquots; about 150 needles) were ground in a mortar in the presence of liquid nitrogen. The resulting fine powder was suspended in 10 ml 80% aq. CH_3OH, allowed to stand for 1 h with continuous stirring, and centrifuged at 2,000×*g* for 15 min to remove soluble phenolics. The pellet was reextracted twice with 80% aq. CH_3OH and then consecutively treated with the following solvents and solutions (10 ml each for 15 min with stirring followed by centrifugations): (i)

$2 \times H_2O$, (ii) $2 \times 0.5\%$ aq. SDS (sodium dodecyl sulphate) [17], (iii) 2×1 M aq. NaCl [18], (iv) $2 \times H_2O$, (v) $4 \times CH_3OH$, (vi) $2 \times$ $(CH_3)_2CO$, and (vii) $2 \times (CH_2CH_3)_2O$. The remaining insoluble whitish material (crude cell walls) was dried overnight at room temperature. In preparative work this procedure was scaled-up by a factor of 100.

Cell wall fractionation. The product of the crude preparation (analytical) was depectinated with 8 ml 30 mM oxalic acid at 100 °C for 3 h [10, 19] or with 8 ml aq. NaH_2PO_4 (200 mM, pH 6.8) at 100 °C for 4 h [20]. After centrifugation ($2{,}000 \times g$, 15 min) the supernatants and pellets were analyzed for the presence of hydrolyzable phenolics (see below). The oxalic acid-depectinated cell wall preparations were further processed as follows: cell walls were suspended in 7 ml sodium acetate buffer (50 mM, pH 5.0) and incubated either with 1 ml purified Driselase (Sigma) at room temperature for 48 h [9, 10] or 50 mg "hemicellulase" (Sigma) at room temperature for 24 h (*cf.* [21]) or alternatively for lignin-carbohydrate preparations in 8 ml 80% aq. 1,4-dioxane at room temperature for 48 h with continuous stirring [22, 23] and the insoluble material further treated with 1,4-dioxane-2 M HCl (9:1) at 70 °C for 8 h [23].

Isolation of insoluble phenolics

Cell walls (crude preparations and insoluble fractions) were suspended in 8 ml hot (80 °C) 1 M aq. NaOH and subsequently allowed to stand for 16 h at room temperature. Solubilized portions from the cell wall fractionation were made alkaline with 10 M aq. NaOH (80 °C) and kept for 16 h at room temperature. Aliquots (0.5 ml) of the hydrolysates were acidified with 50 µl H_3PO_4 and after centrifugation the clear supernatants were directly analyzed by TLC and HPLC.

In preparative work (see above) the acidified hydrolysate was extracted with $(CH_3CH_2)_2O$ and astragalin [24] isolated as follows. The $(CH_3CH_2)_2O$ fraction was dried *in vacuo* and the residue redissolved in 4 ml CH_3OH. This was fractionated on a polyamide column using H_2O and 80% aq. CH_3OH. The latter fraction was evaporated to dryness and the resulting residue redissolved in 4 ml CH_3OH and was chromatographed on a Sephadex LH-20 column using CH_3OH. Elution was monitored continuously by UV absorption at 254 nm and fractions of the eluant showing high UV absorption were collected and subjected to TLC in CAW. The TLC-astragalin bands ($R_f = 0.26$; dark purple in UV light at 350 nm without NH_3 vapour changing to yellow green in UV light with NH_3 vapour) were scraped off and eluted from the cellulose with CH_3OH. The isolated compound was purified on polyamide and Sephadex LH-20 columns as described above.

Identification

The HCAs, *p*-coumaric and ferulic acids, were readily identified from the hydrolysates by chromatographic comparison with reference material (see 'Results and Discussion'). The structure of the isolated astragalin was straightforwardly elucidated from UV/Vis, MS, and ^1H and ^{13}C NMR data (see below).

Chromatography

Thin-layer chromatography (TLC) on microcrystalline cellulose ('Avicel', Macherey-Nagel, Düren, FRG): CAW, $CHCl_3-CH_3COOH-H_2O$ (3:2, H_2O saturated); TAW, toluene-$CH_3COOH-H_2O$ (2:1, H_2O saturated). Gravity column chromatography (CC) on polyamide CC-6 (Macherey-Nagel), 12·2 cm *i.d.*, with H_2O and 80% aq. CH_3OH and Sephadex LH-20 (Pharmacia, Uppsala, Sweden), 67·3 cm *i.d.*, with CH_3OH as solvents. High-performance liquid chromatography (HPLC; Pharmacia-LKB, Freiburg, FRG) on prepacked MN-Nucleosil C_{18} (5 µm, 250·4 mm *i.d.*; Macherey-Nagel); elution system: with a delay time of 0.7 min linear gradient within 20 min from 25 to 60% solvent B ($CH_3OH-CH_3CN-H_2O$, 1:1:1) in solvent A (1.5% H_3PO_4 in H_2O); flow rate at 1.5 ml·min^{-1}; detection at 320 nm. Quantitative calculations were obtained with a Shimadzu (Kyoto, Japan) Data Processor Chromatopac C-R3A using authentic *p*-coumaric acid, ferulic acid, and nicotiflorin – treated in the same way as cell wall preparations – as external standards.

UV/Vis spectroscopy

UV/Vis spectra were recorded in methanolic solution with an UVICON-810 spectrophotometer (Kontron, München, FRG) and astragalin analyzed by using shift reagents as described by Mabry *et al.* [25]. λ_{max} (CH_3OH) nm: 266, 300 shoulder, 349; λ_{max} (+ CH_3ONa) nm: 275, 326, 400; λ_{max} (+ $AlCl_3$) nm: 274, 302, 345, 395; λ_{max} (+ $AlCl_3$ + HCl) nm: 275, 302, 346, 395; λ_{max} (+ CH_3COONa) nm: 274, 303,

378; λ_{max} (+ CH_3COONa + H_3BO_3) nm: 267, 305 shoulder, 353.

NMR and MS spectroscopy

^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded at ambient temperature on a Bruker WM 400 NMR spectrometer locked to the deuterium resonance of the solvent DMSO-d_6. Chemical shifts are reported in ppm relative to TMS. Negative-ion fast atom bombardment (FAB) MS was performed with a Kratos MS 50 mass spectrometer equipped with a Kratos FAB source. Glycerol was used as matrix.

Astragalin (kaempferol 3-O-β-glucopyranoside); see scheme for numbering system. ^1H NMR (DMSO-d_6): δ = 8.014 ["d"; H-2′, H-6′; J(2′-3′) + (J(2′-5′) 8.8], 6.875 ["d"; H-3′, H-5′], 6.420 [d; H-8, J(8-6) 2.0], 6.189 [d; H-6], 5.408 [d; H-1″; J(1″-2″) 7.5], 3.535 [d,d; H-6″A; J(6″A-6″B)(−) 11.2], 3.309 [d,d; H-6″B], 3.25–3.05 (m; H-2″ to H-5″]. ^{13}C NMR (DMSO-d_6): δ = 177.60 (s, C-4), 164.67 (s, C-7), 161.14 (s, C-5), 159.94 (s, C-4′), 156.74, 156.56 (sx2, C-2, C-9), 133.55 (s, C-3), 131.11 (d, C-2′, C-6′), 121.25 (s, C-1′), 115.32 (d, C-3′, C-5′), 104.10 (s, C-10), 101.29 (d, C-1″), 99.02 (d, C-6), 94.06 (d, C-8), 77.51 (d, C-3″), 76.47 (d, C-5″), 74.33 (d, C-2″), 70.02 (d, C-4″), 60.99 (t, C-6″). Negative-ion FAB MS *m/z*: 895 $[2M-H]^-$, 447 $[M-H]^-$, 285 $[M-C_6H_{11}O_5]^-$.

Results and Discussion

In the course of studies on identification of phenolics and their metabolism in Norway spruce (*Picea abies* [L.] KARST.) needles (unpublished) we examined the insoluble material resulting from methanolic extractions of soluble phenolics for the presence of hydrolyzable ester-bound HCAs. This material was thoroughly washed with various solvents as well as NaCl and SDS solutions to obtain crude cell wall preparations [17] which were then treated with aq. NaOH. The latter released both HCA- and flavonoid-like compounds as indicated by TLC. The liberated HCAs were readily identified as *p*-coumaric and ferulic acids (see Scheme) by co-TLC and co-HPLC with reference material (TLC in TAW, typical fluorescence behaviour under UV at 350 nm with and without NH_3 vapour [*e.g.* 26]; HPLC as in Fig. 1). The flavonoid-like compound was isolated on a preparative scale and first subjected to a UV/Vis spectroscopic analysis [25] from which a flavonol 3-glycoside structure could be deduced. The identity of astragalin (kaempferol 3-O-β-

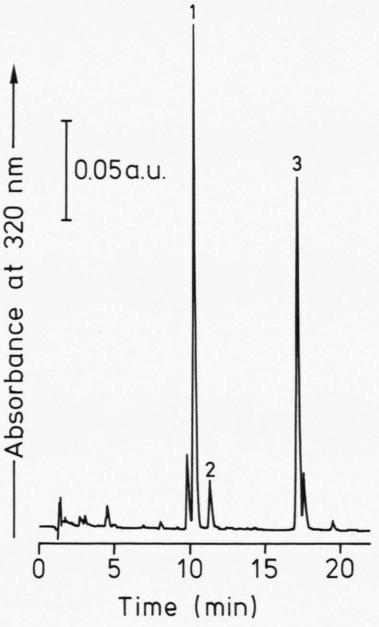
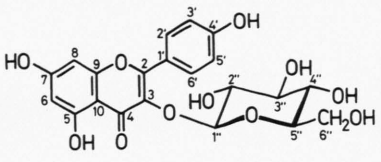
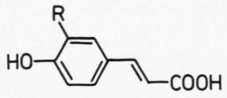


Fig. 1. Diagram of a representative HPLC analysis (see 'Materials and Methods') of cell wall-bound phenolics which were isolated by saponification of a cell wall preparation from Norway spruce needles. Peak identification: 1 = *p*-coumaric acid (R_t = 10.4 min); 2 = ferulic acid (R_t = 11.5 min); 3 = astragalin (kaempferol 3-O-β-glucoside; R_t = 17.3 min).



Kaempferol 3-O-ß-glucopyranoside



R=H p-Coumaric acid
R=OCH_3 Ferulic acid

glucopyranoside; see Scheme) for this compound followed directly from the MS and the ^{1}H and ^{13}C NMR data. The ^{13}C NMR data agree with published data [27]. Quantitative HPLC with reference HCAs and nicotiflorin as external standards gave from 1 g fresh weight of Norway spruce needles about 1.9 μmol *p*-coumaric acid, 1.5 μmol astragalin, and 0.15 μmol ferulic acid, amounting to about 0.1% total cell wall-bound phenolics of needle fresh weight. Fig. 1 shows a diagram of a representative HPLC analysis from one such preparation.

To substantiate the possible cell wall location of the HCAs and astragalin in the Norway spruce needle, various published procedures for cell wall fractionations (see 'Materials and Methods') including depectination, digestion with Driselase and "hemicellulase" (including cellulase activity) as well as lignin-carbohydrate preparations were performed. The results are summarized in Table I and indeed suggest a cell wall location of these compounds. They were detected in only minute quantities in the pectin preparations (oxalate and NaH_2PO_4 treatment) or in (hemi-)cellulose preparations (Driselase and "Hemicellulase" treatment). However, appreciable amounts were found to be released by alkaline hydrolysis of the soluble fractions from the 1,4-dioxane-H_2O and 1,4-dioxane-HCl treatments. This indicates the possible major location of the cell wall-bound HCAs and astragalin in "Björkman-lignin" [28], possibly in "lignin-carbohydrate complexes" [*e.g.* 22, 29].

In summary, the presence of cell wall-bound HCAs described in this paper is in good agreement with pertinent literature (see 'Introduction'). On the other hand, flavonoid glycosides have rarely been found to be associated with cell walls [15, 16] and to the best of our knowledge, this is the first report on a cell wall-bound flavonol glycoside in needles of a conifer. The interesting question on the nature of this flavonoid glycoside-cell wall carbohydrate linkage remains to be elucidated (*cf.* [16]). Studies on this problem as well as a survey of conifers for cell wall-bound phenolics in needles is in progress. In addition, the reactants involved in possible intra- or extracellular acyltransferase reactions in the formation of cell wall-bound phenolics (*cf.* [30]) in Norway spruce needles will be investigated. In this context it is interesting to note that the soluble astragalin in these needles shows a high metabolic activity [31]. We found high concentrations of soluble astragalin as one of the major phenolics in young needles (first year of needle development in spring and summer) and a decrease to low amounts which inversely correlated with a rapid increase in cell wall-bound astragalin together with *p*-coumaric and ferulic acids (unpublished).

Table I. Release by alkaline hydrolysis (aq. NaOH) of *p*-coumaric acid (Coum), ferulic acid (Fer) and astragalin (K3Glc, kaempferol 3-O-β-glucoside) from cell wall fractions of Norway spruce needles obtained by different treatments and subsequent centrifugations (see 'Materials and Methods').

Treatment	Released phenolics [μmol/g fresh weight][a]					
	Supernatant			Pellet		
	Coum	Fer	K3Glc	Coum	Fer	K3Glc
Control[b]	–[c]	–	–	1.89	0.15	1.45
Oxalate	–	–	–	1.72	0.13	1.57
NaH_2PO_4	0.071	–	0.066	1.44	0.11	0.97
Driselase[d]	–	0.055	–	1.38	0.14	1.25
"Hemicellulase"[d]	0.04	–	0.044	1.62	0.15	1.71
1,4-Dioxane-water[d]	0.24	0.032	0.37	1.5	0.11	1.3
1,4-Dioxane-HCl[e]	0.97	0.088	0.21[f]	1.16	0.058	0.13[f]

[a] Mean from two independent experiments (HPLC as in Fig. 1).
[b] Untreated crude cell wall preparation, resuspended in 80% aq. CH_3OH.
[c] – = Not detected.
[d] After oxalate-dependent depectination.
[e] After treatment with 1,4-dioxane-water.
[f] Partly hydrolyzed.

Acknowledgements

Financial supports by the Minister für Umwelt, Raumordnung und Landwirtschaft des Landes Nordrhein-Westfalen (Forschungsprogramm "Luftverunreinigungen und Waldschäden"), the Deutsche Forschungsgemeinschaft (SSP "Physiologie der Bäume" und Heisenberg fellowship), and the Fonds der Chemischen Industrie to D.S. are gratefully acknowledged. The MS spectrum was recorded by Dr. L. Grotjahn, a good colleague and friend, who died on 17th June 1987. We thank Dr. H. Harms (FAL, Braunschweig) for reading the manuscript.

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