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Journal of Chromatography A, 919 (2001) 267–273

JOURNAL OF CHROMATOGRAPHY A

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Reversed-phase and size-exclusion chromatography as useful tools in the resolution of peroxidase-mediated (+)-catechin oxidation products ☆

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Received 20 November 2000; received in revised form 24 March 2001; accepted 2 April 2001

Abstract

The peroxidase-catalysed oxidation of plant phenolics involves one-electron oxidation reactions, and yields unstable mono-radical species, which couple to generate heterogeneous product mixtures of different degrees of polymerisation. One such phenolic susceptible to oxidation by peroxidase is (+)-catechin. Low-pressure chromatography on Sephadex LH-20, using methanol as mobile phase, resolves the main peroxidase-mediated (+)-catechin oxidation products into a dimeric compound (dehydrodicatechin A) and an oligomeric fraction with a polymerisation degree equal or greater than 5. These pure fractions were used to develop rapid high-performance liquid chromatographic methods, both reversed-phase and size-exclusion chromatography for the direct analysis of the peroxidase-mediated (+)-catechin oxidation products. The joint use of both chromatographic systems permitted the qualitative and quantitative identification of the peroxidase-mediated (+)-catechin oxidation products, and can thus be considered as a useful tool for analysing the complex mixtures of natural bioactive plant products synthesized in reactions catalyzed by plant peroxidases. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Catechins; Peroxidase; Phenols; Enzyme inhibitors

1. Introduction

The plant peroxidase (E.C. 1.11.1.7)-catalyzed oxidation of phenols involves one-electron oxidation [1], and yields unstable mono-radical species, $2RH+H_2O_2 \rightarrow 2R^{\cdot}+2H_2O$, which couple to generate dimers, $2R^{\cdot} \rightarrow R_2$. These dimers can be further oxidised by peroxidases, $2R_2H+H_2O_2 \rightarrow 2R_2^{\cdot}+2H_2O$, yielding heterogeneous product mixtures of different degrees of polymerisation, $R_2^{\cdot}+R^{\cdot} \rightarrow R_3$, etc. One of these phenols which may be oxidised by peroxidases is (+)-catechin [2,3]. The peroxidase-mediated oxidation of (+)-catechin yields several products with different polymerisation degrees [2,4]. These (+)-catechin polymers result from repeated condensation reactions between the A ring of one unit and the B ring of another through a mechanism which is known as of ''head to tail'' polymerisation.

Both (+)-catechin, an antimutagenic compound [5], and its oxidation products, especially the vast array of oligomeric compounds, show biological activity. These oligomeric compounds are capable of

☆Presented at the 29th Scientific Meeting of the Spanish Group of Chromatography and Related Techniques, Alcalá de Henares (Madrid), 12–14 July 2000.

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PII: S0021-9673(01)00817-2

inhibiting plant β-glucosidases [6] and bacterial glucosyltransferases [7]. Glucosyltransferase inhibition is especially important in dentistry because such activity contributes to prevention of the development of dental caries in humans by the pathogenic mutant streptococci, such as *Streptococcus mutans* and *S. sobrinus* [7]. Furthermore, some (+)-catechin oxidation products, such as theaflavins and thearubigin, are responsible for the desirable (in tea) or undesirable (in apple and strawberries) browning reactions which occur in post-harvested fruit and vegetables, and their processed plant foods [8,9].

Despite the importance of (+)-catechin oxidation products in medicine and food science, no rapid method has been reported for their detection and quantification by high-performance liquid chromatography (HPLC). Available methods only cover the analysis and detection of the dimers resulting from (+)-catechin oxidation [9,10], and are unable to detect and quantify biologically active (+)-catechin oligomeric compounds. In this report, we describe a rapid HPLC method, using reversed-phase (RP) HPLC and size-exclusion chromatography (HPSEC), for the analysis of the complete set of dimers, trimers, tetramers and oligomers resulting from the peroxidase-mediated (+)-catechin oxidation.

2. Experimental

2.1. Materials

(+)-Catechin, horseradish peroxidase (type II), and 4-methoxy-α-naphthol were obtained from Sigma–Aldrich (Madrid, Spain). Other chemicals and solvents used were of the maximum purity available.

2.2. Determination of peroxidase activity

The spectrophotometric determination of peroxidase activity was carried out in an assay medium containing 1.0 m*M* 4-methoxy-α-naphthol and 0.33 m*M* H_2O_2 in 0.1 *M* Tris–acetate buffer (pH 5.0) by monitoring the increases in absorbance at 593 nm and at 30°C using a Uvikon 940 spectrophotometer (Kontron Instruments, Madrid, Spain). Enzymatic activities were expressed in nkat (nmol of substrate oxidised s^{-1}), an ϵ_{593} of 21000 M^{-1} cm^{-1} being used for the oxidation product [11].

2.3. Oxidation of (+)-catechin and fractionation of the oxidation products

The oxidation of (+)-catechin by peroxidase was performed as described by Weinges et al. [12] with some modifications. For this, 2.5 g (8.6 mmol) (+)-catechin was dissolved in 5 ml acetone and mixed with 100 ml of 50 m*M* sodium citrate buffer (pH 5.6). To this reaction medium, H_2O_2 and horseradish peroxidase were added (in five consecutive additions) up to a final concentration of 0.3% (w/v) and 17 nkat/ml, respectively. After incubation for 24 h at 30°C, a yellow precipitate appeared, which was separated from the dissolution by centrifugation at 3000 g_{max} for 10 min. This precipitate (fraction I) was washed twice with water–acetone (100:5, v/v) and dried on P_2O_5.

The brown soluble phase was then enriched with 500 nkat of horseradish peroxidase and H_2O_2 (final concentration of 0.3%, w/v) and left to react again for 24 h at 30°C. After this time, a brown precipitate was obtained, which was separated from the dissolution by centrifugation at 3000 g_{max} for 10 min. This precipitate (fraction II) was also washed twice with water–acetone (100:5, v/v) and dried on P_2O_5.

Acetone was removed in vacuo from the remaining brown soluble aqueous phase, and this was exhaustively extracted with 1500 ml of ethyl acetate. Solvents from the aqueous and the ethyl acetate phase were removed in vacuo, and the precipitates were dissolved in methanol. The brown precipitate obtained from the ethyl acetate phase was totally soluble in methanol (fraction III).

Finally, the brown precipitate from the aqueous phase was made up in methanol to provide the aqueous fraction (fraction IV), from which a methanol-insoluble white precipitate was separated. This white precipitate was identified by elemental analysis as sodium citrate.

2.4. Purification of dehydrodicatechin A and (+)-catechin oligomers

The main peroxidase-mediated (+)-catechin oxidation products, dehydrodicatechin A and (+)-

catechin oligomers, were purified by low-pressure chromatography on a 70 cm×0.8 cm I.D. Sephadex LH-20 column (Amersham Pharmacia Biotech, Barcelona, Spain) using methanol as mobile phase which was delivered at a flow-rate of 1 ml/min and at room temperature.

Dehydrodicatechin A ($C_{30}H_{24}O_{12}$) (15 mg) was purified with a yield of 90% from fraction I. ^{1}H-Nuclear magnetic resonance (NMR), ^{13}C-NMR and fast atom bombardment (FAB) MS data were in accordance with those reported by Guyot et al. [13].

(+)-Catechin oligomers (15 mg) were purified with a yield of 81% from fraction IV. The δ values in ^{13}C-NMR analysis of this oligomeric fraction closely resembled those obtained for (+)-catechin, therefore it was deduced that the oligomers were composed of repetitive units of (+)-catechin. Furthermore, since these oligomers were resistant to acid hydrolysis in 5 *M* HCl–ethanol (60°C for 3 h), they were not B-type procyanidins, but presumably B-type dehydro-oligocatechins linked by either C–C or C–O–C interflavan bonds [2,14]. When the minimal M_r for this oligomeric fraction was estimated by HPSEC as described in Section 2.5, it was about 1600, indicating that these oligomers were composed of at least five units of (+)-catechin (M_r=290).

2.5. HPLC analyses of (+)-catechin oxidation products

HPLC analysis was carried out in a Waters system (Millipore, Waters Chromatography, Milford, MA, USA) comprising a Model 600 controller, a Model 600 pump, a Rheodyne 7725i manual injector and a Waters 996 photodiode array detector. The data were processed with the Waters Millenium 2010 LC version 2.10 software.

RP-HPLC was carried out at room temperature on a 25 cm×4.6 mm I.D. Waters Spherisorb S5-ODS2 column using a flow-rate of 1 ml/min. Solvent A was 2.5% acetic acid in water and solvent B was acetonitrile. A linear gradient from 0 to 10% B in 5 min, from 10 to 30% B in 20 min, and from 30 to 50% B in 20 min was used.

HPSEC was carried out at room temperature on a 30 cm×7.8 mm I.D. TSK-Gel G2500HR column (TosoHaas, Tosoh, Montgomeryville, PA, USA) using dimethylformamide as eluent at a flow-rate of 1 ml/min, and a set of polystyrene M_r standards (Sigma–Aldrich).

All samples were filtered through 0.45-μm membrane filters (Scharlau, Barcelona, Spain), and a 20 μl volume of each sample (fractions I–IV) in methanol was injected and chromatographed under the above conditions. Due to the different λ_{max} of the (+)-catechin oxidation products (Table 1), chromatographic profiles were obtained as Maxplot (λ_{max} chromatograms), defined in the Waters Millenium software as the chromatographic channel where each data point is the absorbance maximum of the spectrum acquired at a given point in time. Semi-quantification of (+)-catechin oxidation products was performed at 280 nm using (+)-catechin as standard. The minimum detectable quantity of (+)-catechin in HPSEC was found to be approximately 0.03 μg with a signal-to-noise ratio of 2 at 0.0005 AUFS. The precision of the analysis was assessed by replicate analyses of (+)-catechin, and then the variations were calculated. The response for a series of five injections of 5.0 μg (+)-catechin resulted in relative standard deviations of 0.5% for the retention time (t_R) and 3.6% for the peak area.

2.6. Other analyses

^{1}H- and ^{13}C-NMR analyses were performed in

Table 1
UV–Vis characteristics of (+)-catechin and the peroxidase-mediated (+)-catechin oxidation products in the two HPLC systems used

Compound	λ_{max} (nm) in RP HPLC (acetic acid/acetonitrile)	λ_{max} (nm) in HPSEC (dimethylformamide)
(+)-Catechin	235.9, 279.4	281.8
Dehydrodicatechin B4	233.5, 279.4	
Dehydrodicatechin A	233.5, 257.0, 279.4, 382.6	281.8, 371.8, 447.5
(+)-Catechin trimer	234.7, 257.0, 280.6, 363.6	281.8, 362.7, 442.6
(+)-Catechin oligomers	238.2, 280.6, 383.8	283.0, 381.4, 431.8

$C^2H_3O^2H$ at room temperature in a Variant Unity (Palo Alto, CA, USA) at 300 MHz. FAB-MS analyses were performed in the negative mode (3-nitrobenzyl alcohol matrix) using an Autospec 5000 VG instrument (Manchester, UK). Elemental analyses were performed in a Carlo Erba CNHS-O EA1108 elemental analyzer (Milan, Italy).

3. Results and discussion

3.1. Use of RP-HPLC and HPSEC

RP-HPLC analyses using a C_{18} column of the peroxidase-mediated (+)-catechin oxidation products of an oligomeric nature, isolated from fraction IV and previously purified by low-pressure chromatography on Sephadex LH-20, yielded a hunchback profile which hinders both qualitative and quantitative analysis by HPLC. This hunchback profile can be avoided when this oligomeric fraction is analysed by HPSEC on a TSK-Gel G2500HR column using dimethylformamide as mobile phase.

HPSEC was useful not only for the analysis of (+)-oligomers, but also for the simultaneous resolution of other (+)-catechin oxidation products of a lower polymerisation degree. Thus, in this system, (+)-catechin oligomers (retention time, t_R=5.36 min) can clearly be separated from a (+)-catechin dimer, such as dehydrodicatechin A (t_R=7.51 min), and from (+)-catechin itself (t_R=8.54 min). However, HPSEC is unable to resolve (+)-catechin dimers, such as dehydrodicatechin A and dehydrodicatechin B4 (Fig. 1), due to their similar M_r values. For this reason, both RP-HPLC (on a Spherisorb S5-ODS2 column) and HPSEC (on a TSK-Gel G2500HR column) were used together to analyse and to dissect the complex product mixtures which result from the peroxidase-catalyzed (+)-catechin oxidation.

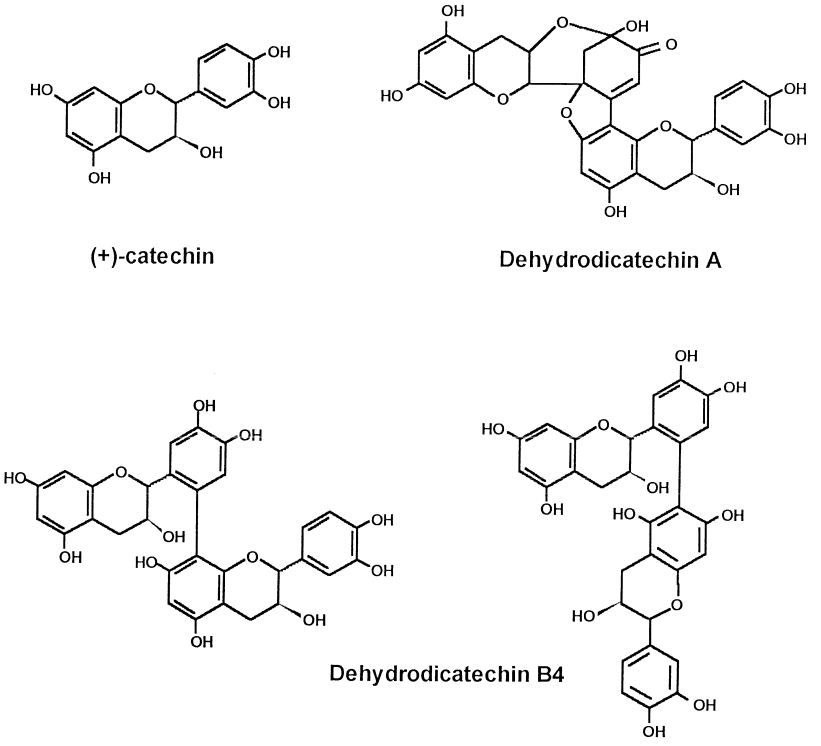

Fig. 1. Structures of (+)-catechin, dehydrodicatechin A and possible structures of dehydrodicatechin B4.

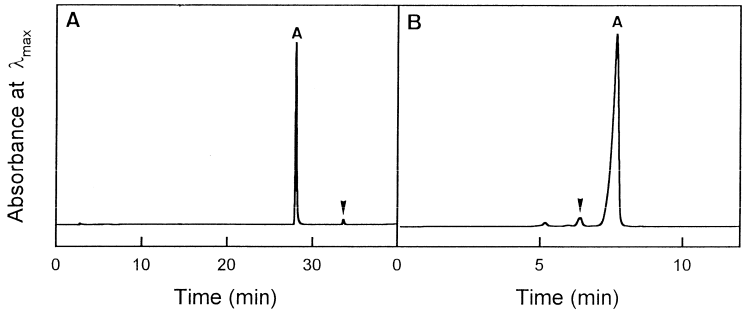

Fig. 2. HPLC chromatogram on a Spherisorb S5-ODS2 column (A) and on a TSK-Gel G2500HR column (B) of the peroxidase-catalyzed (+)-catechin oxidation products present in fraction I. HPLC conditions are described in the Experimental section. A: Dehydrodicatechin A. Arrowhead: (+)-catechin trimer.

3.2. HPLC determination of (+)-catechin oxidation products in fraction I

Analysis by RP-HPLC (Fig. 2A) and HPSEC (Fig. 2B) of fraction I indicated that this fraction is mainly composed (up to 91%) of dehydrodicatechin A. It was also possible to detect in this fraction a smaller (3%) amount of a second compound (Fig. 2A and B; arrowhead). Attempts to estimate the M_r for this compound by HPSEC pointed to a mean M_r value of about 950, suggesting that this product is the (+)-catechin trimer described by Hamada et al. [4], for which they reported an m/z of 865 for the $[M+H]^+$ species in FAB-MS. Differences in the M_r for the two compounds may be due to the differences in hydrodynamic behaviour (determined by the molecular geometry) between styrenes and (+)-catechins in this HPLC system. Since this compound showed a UV spectrum containing the main features of (+)-catechin (Table 1), these results together suggest that this compound is putatively formed by three repetitive units of (+)-catechin.

3.3. HPLC determination of (+)-catechin oxidation products in fraction II

Analysis by RP-HPLC (Fig. 3A) and HPSEC (Fig. 3B) of fraction II indicated that this fraction is composed of (+)-catechin oligomers (69%), the putative (+)-catechin trimer (13%), dehydrodicatechin A (8%) and unreacted (+)-catechin (6%). In this fraction, it was also possible to detect small

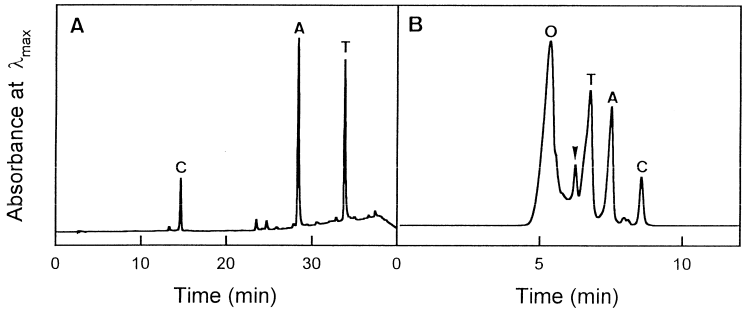

Fig. 3. HPLC chromatogram on a Spherisorb S5-ODS2 column (A) and on a TSK-Gel G2500HR column (B) of the peroxidase-catalyzed (+)-catechin oxidation products present in fraction II. HPLC conditions are described in the Experimental section. O: (+)-Catechin oligomer. T: (+)-catechin trimer. A: Dehydrodicatechin A. C: (+)-Catechin. Arrowhead: (+)-catechin tetramer.

amounts of a fifth compound (Fig. 3A and B; arrowhead). Attempts to estimate the M_r for this compound by HPSEC using dimethylformamide as mobile phase pointed to a mean M_r value of about 1190. The value of M_r found for this compound suggests that this product is probably the (+)-catechin tetramer described by Hamada et al. [4], although they reported a m/z of 1153 for the $[M+H]^+$ species in FAB-MS. Again, differences in the M_r for the two compounds may be due to the different hydrodynamic behaviours (determined by the molecular geometry) of styrenes and (+)-catechins in this HPLC system.

3.4. HPLC determination of (+)-catechin oxidation products in fraction III

Analysis by RP-HPLC (Fig. 4A) and HPSEC (Fig. 4B) of fraction III indicated that this fraction is composed of (+)-catechin oligomers (21%), the putative (+)-catechin trimer and tetramer (21% together), dehydrodicatechin A (8%) and unreacted (+)-catechin (48%). In this fraction, it was also possible to detect by RP-HPLC (Fig. 4A) minor amounts of a sixth compound (arrowhead). Attempts to estimate the nature of this compound from both its retention time (Fig. 4A) and UV–visible spectrum (Table 1) in RP-HPLC indicate that it closely matches dehydrodicatechin B4 [13]. Dehydrodicatechin B4 is the precursor of dehydrodicatechin A, which is derived from the former through a single step enzymatic oxidation followed by an internal stabilisation of the radical species via two intramolecular nucleophilic additions.

3.5. HPLC determination of (+)-catechin oxidation products in fraction IV

Finally, analysis by RP-HPLC (Fig. 5A) and HPSEC (Fig. 5B) of fraction IV indicated that this fraction is mainly composed of (+)-catechin oligomers (83%). In this fraction, both the putative (+)-catechin trimer and tetramer (10% together) may also be detected (Fig. 5B).

4. Conclusion

The present results suggest that the joint use of both RP-HPLC (on a Spherisorb S5-ODS2 column) and HPSEC (on a TSK-Gel G2500HR column) permits the qualitative and quantitative identification of dimers (dehydrodicatechin A and dehydrodicatechin B4), trimers, tetramers and oligomers resulting from the peroxidase-mediated (+)-catechin oxidation.

Acknowledgements

This work was partially supported by a grant from the Comisión Interministerial de Ciencia y Tecnología (Spain, project ALI 1018/95) and from the Ministerio de Educación y Cultura (Spain, project PB97-1042).

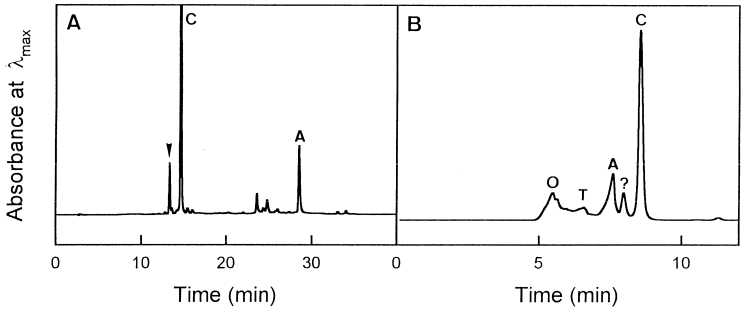

Fig. 4. HPLC chromatogram on a Spherisorb S5-ODS2 column (A) and on a TSK-Gel G2500HR column (B) of the peroxidase-catalyzed (+)-catechin oxidation products present in fraction III. HPLC conditions are described in the Experimental section. O: (+)-Catechin oligomer. T: (+)-Catechin trimer. A: Dehydrodicatechin A. C: (+)-Catechin. Arrowhead: Dehydrodicatechin B4. ?: Unidentified compound.

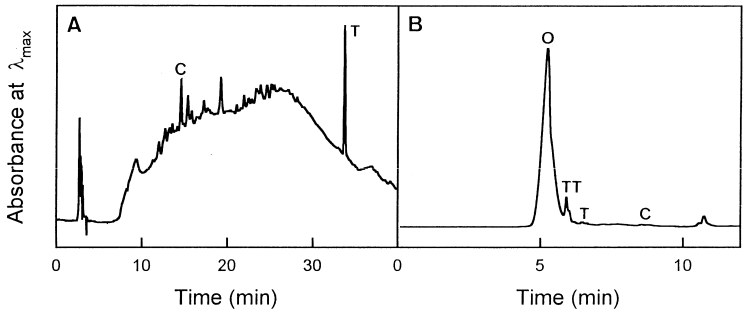

Fig. 5. HPLC chromatogram on a Spherisorb S5-ODS2 column (A) and on a TSK-Gel G2500HR column (B) of the peroxidase-catalyzed (+)-catechin oxidation products present in fraction IV. HPLC conditions are described in the Experimental section. C: (+)-Catechin. O: (+)-Catechin oligomer. T: (+)-Catechin trimer. TT: (+)-Catechin tetramer.

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