Separation of High Molecular Weight Sorghum Procyanidins by High-Performance Liquid Chromatography

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A method was developed for using high-performance liquid chromatography (HPLC) to fractionate high molecular weight procyanidins isolated from sorghum grain. Procyanidins were eluted from a short reversed-phase (C_{18}) column by a combination of linear and step gradients and a fast flow rate. Retention times generally increased with the relative degree of polymerization. Phytic acid included in the mobile phase to chelate metal ions in the silica matrix prevented on-column reactions of these materials. This HPLC method is applicable to the study of procyanidin stability and the interaction of procyanidins with protein.

Procyanidins, commonly called condensed tannins, are found in the seed coat of many varieties of sorghum, especially those known to be bird resistant. These procyanidin oligomers can have from 2 to 40 units (Williams et al., 1983) linked as a straight chain or with branching (Mattice and Porter, 1984). High molecular weight procyanidins are capable of binding to proteins and therefore have biological significance, possibly as part of the plant's defense system.

Study of these higher molecular weight procyanidins has been limited by difficulties in obtaining them as homogeneous fractions. Various separation techniques have been used to isolate procyanidins. These include conventional column chromatography, high-performance liquid chromatography (HPLC), and countercurrent chromatography (Putman and Butler, 1985). Examples of solid supports used in column chromatography to separate dimers, trimers, and tetramers are Fractogel TSK (Derdelinckx and Jerumanis, 1984), Sephadex G-25 (Feeny and Bostock, 1968; Porter and Wilson, 1972), cellulose (Karchesy and Hemingway, 1980), and Sepharose CL-6B (McGrath et al., 1982). Procyanidins with five and six units were separated by repeated chromatography using Sephadex LH-20 and MCI-gel CHP 20P (Morimoto et al., 1986). HPLC with Spherisorb Hexyl (Lea, 1980) and Zorbax CN (Wilson, 1981) columns separated procyanidins up to pentamers and heptamers, respectively.

Separation of higher polymers into homogeneous fractions has not been successful. When polymers were eluted from a μBondapak C_{18} column with 2.5% acetic acid and methanol, they gave a broad band (McMurrough, 1981). Polymers eluted from Lichrosorb Si 60 gave broad bands not returning to the base line (Glennie et al., 1981). Lea (1980) reported that oxidized polymeric tannins eluted en masse with a sharp increase in gradient steepness.

This paper describes a method to fractionate high molecular weight procyanidins from sorghum by HPLC. Applications of this method are also discussed.

MATERIALS AND METHODS

HPLC was performed on a Varian 5000 liquid chromatograph combined with a Varian UV-50 variable-wavelength detector (Palo Alto, CA). For later work, a Hewlett-Packard 1040A diode array detection system (Palo Alto, CA) was used.

Methanol and acetonitrile were chromatography grade, and solvents prepared for HPLC were filtered before use. The column, 5 cm × 4.6 mm, was packed with Synchropak RP-P300 (Synchrom Inc., Lafayette, IN), a C_{18} support with 300-Å pores, and 10-μm particles.

Samples were filtered before injection with centrifugal microfiltration units (Bioanalytical Systems Inc., West Lafayette, IN). Filters were regenerated cellulose with 0.45-μm pores.

Procyanidins B-2, B-3, and B-9 were provided by Prof. E. Haslam, and the peptide from mouse submaxillary glands was donated by Dr. D. Carlson. Purified oligomeric procyanidins from sorghum (grain) Br64 and IS 8768 (grown at the Purdue University Agronomy Farm, under the direction of Dr. J. Axtell) were prepared by the method of Hagerman and Butler (1980). Phytic acid (sodium salt) was obtained from Sigma. All other chemicals were reagent grade.

The gradient developed for separating purified oligomeric procyanidins was a combination of linear and step gradients. Solvent A was 1 mM phytic acid in water (or 1 mM EDTA or water)–glacial acetic acid (1000:1), and solvent B was methanol–water–acetic acid (800:200:1). The gradient was linear from 5-15% B over 15 min followed by isocratic elution at 15% B for 5 min and then a step gradient eluting for 5 min at 20, 30, 40, 50, and 100% B. The flow rate was 3.8 mL/min. When the diode array detector was used, acetonitrile replaced methanol in solvent B and the flow rate was reduced to 3 mL/min. These modifications were necessary to minimize back-pressure created by the small inlet tubing of the detector.

An assay with 4-(dimethylamino)cinnamaldehyde (DAC) was used to detect terminal units of procyanidins. DAC, similar to vanillin, specifically reacts with flavonoids possessing free meta-oriented hydroxy groups in the A ring (McMurrough and McDowell, 1978). In its reaction with procyanidins, DAC complexes only with terminal units. The assay using DAC instead of vanillin is more sensitive, detecting catechin in the range of 4–10 μg. The procedure was adapted from McMurrough and McDowell (1978) using 1% DAC in 25% HCl–methanol and measuring the absorbance at 640 nm after 1.25 min. The upper units of procyanidins were detected by the proanthocyanidin assay (Butler, 1982).

The relative degree of polymerization (RDP) of a procyanidin fraction was calculated by dividing the total number of monomeric units by the number of terminal units. The total monomeric units was a sum of upper units and terminal units (determined by the proanthocyanidin and DAC assay, respectively). Purified samples of oligomeric procyanidin and catechin were assayed to standardize this calculation (Putman and Butler, 1985).

Values obtained for RDPs are not meant to specify the number of units in an oligomer. They are crude values that are useful in comparing the average chain length of procyanidin fractions. The proanthocyanidin assay is largely responsible for the deviation between the calculated RDP and the actual degree of polymerization. Hydrolysis of proanthocyanidins in this assay is not quantitative, and the yield varies with the type of linkage between units (Porter et al., 1986).

RESULTS AND DISCUSSION

Separation of previously purified procyanidin fractions was achieved by reversed-phase HPLC using a combination of linear and step gradients (Figure 1). Lower mo-

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Table I. Retention Times of Procyanidin Standards

procyanidin	T_r, min
B-3 (catechin dimer)	1.15
B-2 (epicatechin dimer)	3.20
B-9 (epicatechin trimer)	7.60

lecular weight procyanidins eluted during the linear part of the gradient. Catechin and epicatechin (monomers) were not retained by the column, eluting with the solvent front, and dimers and a trimer eluted during the linear portion of the gradient with retention times shown in Table I. Larger molecular weight procyanidins eluted during the step gradient. Peaks eluting at 20% B (27.1 min), 30% B (31.8 min), and 40% B (36.9 min) (Figure 1B) were collected and analyzed by the proanthocyanidin and DAC assays. RDPs were determined to be 10, 11, and 13, respectively. These data indicate that elution proceeds according to increasing molecular weight.

Parts A and B of Figure 1 represent procyanidin fractions with different RDPs obtained from a Sephadex LH 20 column similar to that described by Hagerman and Butler (1980). The average RDPs are 2 and 6 for parts A and B of Figure 1, respectively. As expected, the fraction with the lower RDP has more components eluting early in the gradient while the fraction with an RDP of 6 has components mostly in the later part of the gradient. This figure also demonstrates the heterogeneity of procyanidin fractions from a Sephadex LH 20 column and the resolving power of HPLC. A single fraction eluted from Sephadex LH 20 was separated into several components by HPLC.

The mechanism of separation in reversed-phase (RP) HPLC depends on both adsorption and desorption. Procyanidins can be adsorbed to the stationary phase through both silanophilic and solvophobic interactions (Nahum and Horvath, 1981). The phenolic hydroxyls of the procyanidin can form hydrogen bonds with silanol groups on the stationary phase (silanophilic interactions), and the aromatic rings can interct with the alkyl chains of the stationary phase (solvophobic interactions). Thus, procyanidins are tightly bound to the column.

Desorption of a solute in reversed-phase chromatography is achieved by increasing the concentration of organic solvent in the mobile phase (Regnier, 1983). In the elution of procyanidins, an additional mechanism was affecting desorption because changing from a linear gradient to a step gradient changed the elution from a broad band to a series of discreet peaks. This other mechanism is solute–solvent interactions (Snyder et al., 1983). A similar phenomenon was observed in RP-HPLC of proteins (O'Hare et al., 1982). Because a faster flow rate and steeper gradient gave better recovery, the changing mobile phase was thought to alter the protein's conformation, changing its affinity for the stationary phase to allow desorption. Solvent-induced aggregation was also suggested.

In this RP-HPLC separation of oligomeric procyanidins, the rapid increase in concentration of organic solvent in the mobile phase due to a fast flow rate and a step gradient may cause a conformational change or an aggregation of procyanidins leading to desorption. A faster flow rate makes this change even more abrupt, enhancing the conformational change and giving better resolution.

This desorption is not a nonselective process of pulling molecules away from the stationary phase. It is dependent on the solvent concentration. When peaks eluting at 20, 30, and 40% B were collected and then reinjected, they gave the original peaks plus peaks later in the gradient (Figure 2). The later peaks may be explained by on-column reactions (to be addressed shortly) and oxidative polymerization during handling. If the sharp change in

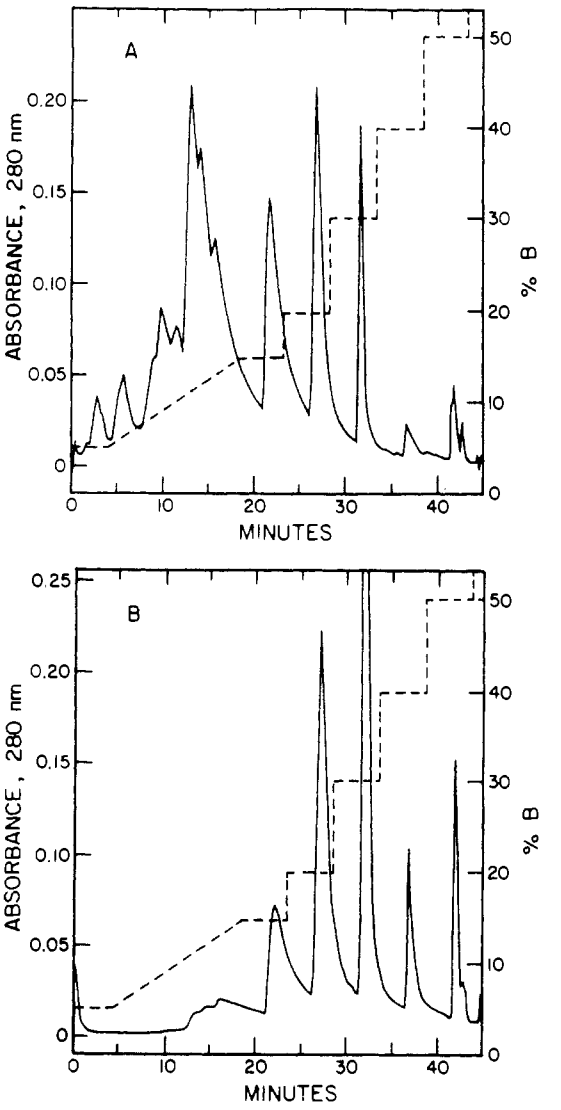

Figure 1. HPLC of purified procyanidin fractions with different RDPs. Fractions with RDPs of 2 (A) and 6 (B) were separated with 1 mM EDTA–acetic acid (1000:1) as solvent A and methanol–water–acetic acid (800:200:1) as solvent B. The flow rate was 3.5 mL/min. Gradient is shown by dotted line. Procyanidins eluting at 20% B (27.1 min), 30% B (31.8 min), and 40% B (36.9 min) had RDPs of 10, 11, and 13, respectively.

solvent was nonselectively removing procyanidin molecules by shear forces or viscosity changes, then the reinjected fractions would also have peaks at earlier steps in the gradient.

To examine the components of a sorghum procyanidin fraction that eluted during the step gradient, diode array detection was used (Figure 3). Absorbance spectra taken at the up slope, apex, and down slope of three major peaks (22, 27, and 32 min) were superimposed in Figure 3a–c as an indication of the homogeneity of each peak. Spectra taken at the apex of each peak were superimposed in Figure 3d. These UV spectra were nearly identical, suggesting that the difference between these peaks is not in the oxidation state of the heterocyclic ring or in the hydroxylation pattern. These properties are known to affect the UV spectra of flavonoids. Differences in chain length may be the major factor causing separation.

The problem of on-column reactions is most often caused by trace metal impurities present in the silica matrix of chromatographic supports (Verzele and Dewaele, 1981). To counter the effects of trace metals, ethylenediaminetetraacetic acid (EDTA) or phytic acid was in-

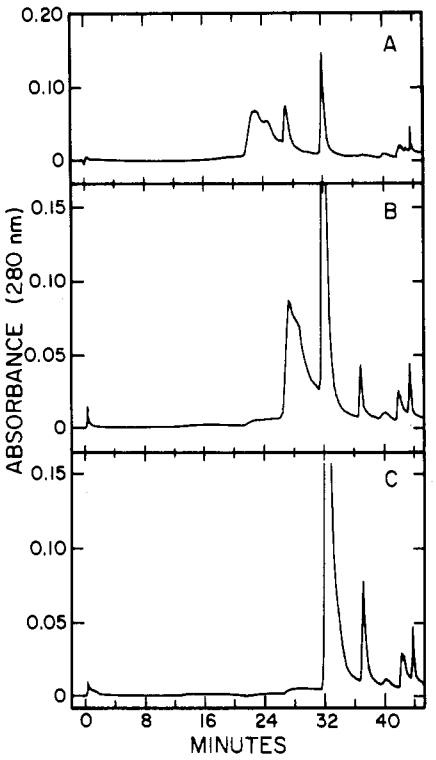

Figure 2. HPLC of reinjected peaks with 1 mM phytic acid–acetic acid (1000:1) as solvent A and methanol–water–acetic acid (800:200:1) as solvent B. Peaks eluting at 20, 30, and 40% B (see Figure 1B) were collected from multiple HPLC separations of a crude extract of tannin from Br64 sorghum. Collected peaks were reinjected. A–C show the separation of reinjected peaks that originally eluted at 20, 30, and 40% B, respectively.

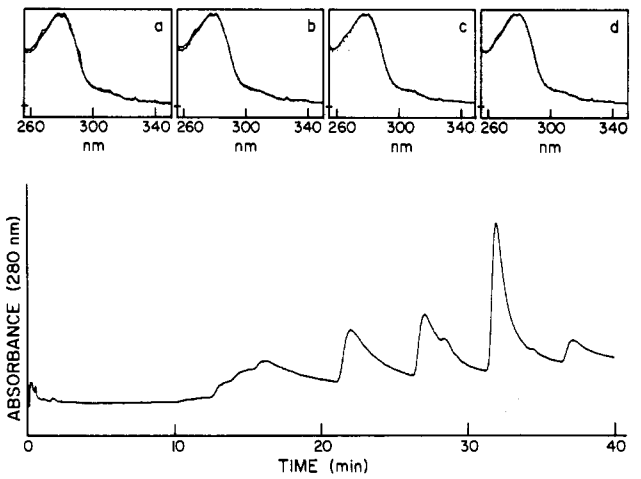

Figure 3. HPLC of purified procyanidin fraction by diode array detection. Solvent A was water–acetic acid (1000:1) and solvent B was acetonitrile–water–acetic acid (800:200:1). The flow rate was 3.0 mL/min. In a–c absorbance spectra from the up slope, apex, and down slope of peaks at 22 min (a), 27 min (b), and 32 min (c) were superimposed. In d, spectra taken at the apex of these three peaks were superimposed.

cluded in solvent A to chelate metal ions that may reside on the column. Leadbetter and Allen (1985) showed that EDTA was bound to reversed-phase packing material, indicating that it was chelating metal ions on the support. Phytic acid is preferred over EDTA as a chelator because the complex it forms with Fe^{3+} lacks the open iron coordination site found in other iron chelates that may catalyze hydroxyl radical formation (Graf et al., 1984).

The effectiveness of phytic acid and EDTA in preventing on-column oxidation was compared by reinjecting

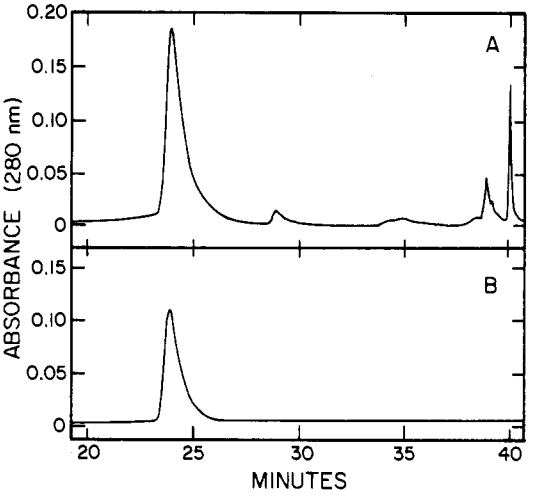

Figure 4. HPLC of reinjected peaks to compare the effects of EDTA (A) and phytic acid (B) in the solvent. Purified procyanidin was separated by HPLC with solvents containing EDTA or phytic acid. The peak eluting at 30% B was collected and reinjected, eluting with the respective solvent. In A, solvent A was 1 mM EDTA–acetic acid (1000:1) and in B, solvent A was 1 mM phytic acid–acetic acid (1000:1). Solvent B in both cases was methanol–water–acetic acid (800:200:1).

peaks eluted at 30% B (Figure 4). When EDTA was included in solvent A, small peaks at 40 and 50% B appeared in addition to the reinjected peak at 30% B. Phytic acid was more effective than EDTA because reinjection of the peak eluted at 30% B showed no additional peaks. Although phytic acid was included in the solvent for the reinjected peaks in Figure 2, on-column reactions appeared to be present. The sample used in Figure 4 was a purified procyanidin fraction, whereas the peaks in Figure 2 were from the separation of a crude extract of procyanidins. It is likely that other material present in the crude extract contributed to the formation of oxidation products.

This HPLC method has several applications in the study of oligomeric procyanidins. Although it does not give a detailed analysis of structure as NMR would, it provides additional information about procyanidin fractions. It is useful for monitoring large-scale preparations of purified procyanidins and for examining the stability of procyanidins under various conditions. For example, incubating a procyanidin fraction in 0.1% acetic acid for 2 days produced changes in the chromatographic profile, but in the presence of 1 mM ascorbic acid, dithiothreitol, or phytic acid no significant changes occurred.

In addition to purified procyanidin samples, crude extracts of procyanidins may be separated by this method. They contain more components that elute early in the gradient. Adjusting the gradient to start at 0% B improved resolution of these components. Analysis of crude procyanidin extracts is useful for comparing different cultivars of sorghum as to the types of phenolics they contain. For example, chromatograms of extracts from high-tannin sorghums are dominated by peaks eluting during the step gradient, whereas most of the components in low-tannin sorghum extracts elute during the linear part of the gradient.

Investigation of the specificity of the protein binding activity of oligomeric procyanidins is also possible with this technique. A peptide generated from a mouse submaxillary gland glycoprotein (Asquith et al., 1987) was combined with a mixture of high-MW procyanidins in aqueous solution to form insoluble complexes. HPLC of the original procyanidin solution and the supernatant following precipitation showed that there was a 74% decrease in the

area of the peak eluting at 40% B. Those peaks eluting at 20 and 30% B decreased by only 40 and 42%, respectively. The protein selectively bound the larger polymers to form insoluble complexes. This result demonstrates that the high-MW procyanidins eluting at 40% B are not too large to be biologically significant.

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Registry No. EDTA, 60-00-4; phytic acid, 83-86-3.

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