Proanthocyanidins in Wheat Bran

J. A. McCALLUM[1] and J. R. L. WALKER[2]

ABSTRACT

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Commercial wheat bran was found to contain low levels of catechin and di-, tri-, and oligomeric proanthocyanidins soluble in aqueous acetone. Earlier attempts to detect these compounds in wheat appeared to have failed because of interference from methoxyhydroquinone glycosides. The oligomeric proanthocyanidins contained mostly prodelphinidin and some procyanidin units, whereas the dimeric proanthocyanidins may also contain some propelargonidin units. Procyanidin B3 (catechin-[4α→8]-catechin) and prodelphinidin B3 (gallocatechin-[4α→8]-catechin) were isolated and characterized. The contribution of these compounds to seed coat color is discussed.

Proanthocyanidins (PAs) are colorless phenolic oligomers or polymers based on flavan-3-ol units and, like the closely related leuco-anthocyanidins, they are converted to colored anthocyanidins by strong acids. PAs consist of dimeric to polymeric chains of C(4)→C(8) linked 2,3-trans- and 2,3-cis-flavan-3-ol units (Fig. 1); additionally there may be C(4)→C(6) linkages that permit branching of polymers. The hydroxylation pattern of the A-ring is usually 5,7-hydroxy and that of the B-ring is usually either 3',4'-OH, a procyanidin (PCy unit) or 3',4',5'-OH, a prodelphinidin (PD unit) (Foo and Porter 1980). Due to their high degree of hydroxylation, PAs are very polar and readily form insoluble complexes with protein and carbohydrate by hydrogen bonding (Spencer et al 1988). PAs also have the biologically important ability to cross-link and precipitate proteins (hence their name "condensed tannins").

Flavan-3-ols and PAs have been shown to have a variety of effects on plant growth and development such as determining the germinability and vigor of lettuce seeds (Buta and Lusby 1986). Cereal seed coats have been reported to contain a variety of flavonoids (Collins 1987) and these have been found to have significant effects on the quality of cereal grains. Sorghum contains high levels (up to 5% dry weight) of proanthocyanidin polymers (Brandon et al 1982), and these may significantly reduce the nutritional quality of some cultivars. Barley has been shown to contain the monomeric flavan-3-ols (+)catechin and (+)gallo-catechin and a variety of procyanidin and prodelphinidin oligomers (Brandon et al 1982, Gujer et al 1986). Histological (Cochrane and Duffus 1983) and physiological (Jacobson and Corcoran 1977) studies of developing barley grains have provided some evidence that PAs in the seed coat may play an important role in restricting entry of water and oxygen and thus influence germinability.

PAs have been found in the pericarp of immature seeds of brown and red rice varieties, and it was suggested by Nagao et al (1957) that the seed coat pigments of the mature grains are phlobaphenes formed by oxidation of the PAs. Similarly, for immature wheat grain, Miyamoto and Everson (1958) observed that there is an apparent correlation between concentration of PAs and mature seed coat color: they suggested therefore that the well-known association between grain redness and dormancy is due to inhibitory effects of the PAs. This hypothesis was challenged by Gordon (1979), who examined changes in flavanol content during grain maturation and failed to observe any relationship between flavanol content and dormancy. Both of these studies used colorimetric methods, and no isolation of specific compounds or other validation of assays was attempted.

In an earlier study by Pollock et al (1960) utilizing colorimetric methods, the PA content of several cereal grains was compared and PAs could not be detected in mature wheat grain.

PAs in the seed coat of winter wheats may have particular significance as protective agents because they are resistant to microbial degradation (Harborne 1985), and their presence may confer some protection to overwintering seeds from soil microorganisms.

PAs may also be involved in controlling dormancy and germination (Meredith and Pomeranz 1985), and a study of the inheritance of germination response to PAs in excised wheat embryos suggested that this is controlled by a single gene (Stoy and Olsen 1980). Therefore, PAs and other phenolics may influence germination in some situations, but our understanding of their role is still limited by lack of understanding of their structures, concentration, and localization in the grain.

The work to be reported here is part of a wider study (McCallum 1989) of the biochemistry of phenolic compounds in wheat grain; PAs were of interest because of their possible effects on germinability, dormancy and nutritional quality.

MATERIALS AND METHODS

Materials

Coarse commercial bran milled from pooled red-grained wheat cultivars Rongotea (70%) and Oroua (30%) was supplied by D.H. Brown & Son, Flour Millers, Christchurch, N.Z. All organic solvents were distilled before use. Delphinidin, pelargonidin, and (−)epicatechin were obtained from Fluka AG (Switzerland); (+)catechin was obtained from Sigma Chemicals (St. Louis, MO). Purified quince procyanidin was provided by L. J. Porter, Chemistry Division, D.S.I.R., Petone, N.Z.

Methods

Analytical thin-layer chromatography (TLC) was performed on Schleicher and Schull Avicel cellulose sheets, whereas paper chromatography (PC) of anthocyanidins was carried out in descending mode on Toyo 51A chromatography paper. The following solvent systems were used: A, tert-butanol/glacial acetic acid/water, 3:1:1 (=TBA); B, 6%, v/v, acetic acid (=HAc); C, concd HCl/glacial acetic acid/water 3:30:10 (=Forestal). Flavanols were visualized by spraying chromatograms with vanillin-HCl (4% vanillin in ethanol/concd HCl 4:1) followed by warming (Markham 1982). Authentic samples of catechin, cyanidin, and delphinidin were included for chromatographic comparisons.

Bran (600 g) was ground in a Casella blade mill to pass a 1.5-mm screen. The ground bran was placed in a large chromatography column (20 × 5 cm) and percolated with 4 L of 70% aqueous acetone containing 0.1% ascorbic acid to extract PAs. Combined extracts were clarified by vacuum filtration through Toyo CF paper and fractionated according to previously published methods (Czochanska et al 1980, Foo et al 1982).

The filtered extracts were saturated with NaCl to salt out the acetone and the upper solvent phase was removed. This acetone

phase was then extracted with three successive 250-ml portions of the aqueous phase of NaCl-saturated 70% aq acetone containing 0.1% ascorbate to prevent auto-oxidation. The acetone was then removed in vacuo at 25°C and an equal volume of water was added. This aqueous solution was then extracted, first with three successive portions of petroleum ether (40–60°C) to remove any lipid material and then with three successive portions of ethyl acetate to extract low molecular weight PAs. The combined ethyl acetate extracts (EA1) were dried over anhydrous sodium sulfate and reduced to dryness at 25°C in vacuo. Finally, the remaining aqueous phase containing the PA oligomers was dialyzed in the dark, under an N_2 purge, against three succesive 5-L portions of 0.1% ascorbic acid and then freeze-dried (Aq1).

The ethyl acetate fraction (EA1) containing the low molecular weight monomers and dimers was redissolved 95% ethanol, applied to a column (100 cm × 2.5 cm) of Sephadex LH-20, which previously had been equilibrated with 95% ethanol and then eluted with the same solvent. Collected fractions (50 ml) were examined for flavanols by TLC using solvent B and vanillin/HCl spray as the location reagent.

The aqueous (Aq1 or "oligomeric") fraction was also purified by chromatography on Sephadex LH-20. The freeze-dried, dialyzed extract was redissolved in 30% aq methanol containing 0.1% ascorbate and applied to a 150 × 16 mm column of LH-20 equilibrated with the same solvent. The column was eluted first with 1.5 L of 30% aq methanol containing 0.1% ascorbate, and then the PAs were eluted with 200 ml of 70% aq acetone. Acetone was removed at 25°C in vacuo, and the resulting aqueous residue was freeze-dried (Aq2).

Acid hydrolysis of fractions and purified compounds was performed in screw-capped vials according to standard methods (Harborne 1984). Samples, in 0.5 ml of methanol, were heated with 3 ml of n-butanol/concd HCl (95:5) in a boiling water bath for 30 min. The hydrolysates were examined by PC or TLC in solvent system C with authentic delphinidin, pelargonidin, and quince procyanidin hydrolysate as standards.

For identification of anthocyanidins separated by PC, the spots were cut out and their absorption spectra recorded directly against paper blanks in a Pye Unicam SP1800 spectrophotometer. For determination of PCy/PD ratios, spots corresponding to cyanidin and delphinidin were cut out from PCs and eluted twice with methanol containing 1% concd HCl. Extracts were concentrated in vacuo, and the cyanidin and delphinidin were estimated by measuring absorbance at 550 and 560 nm, respectively.

## Acid Degradation of PAs in Presence of Phloroglucinol

In order to identify monomeric PA components, samples of PAs (usually 500 μg) were incubated with an equal weight of phloroglucinol in 200 μl 0.1 M methanolic-HCl at room temperature; this treatment degrades PA oligomers to yield the PA-monomer plus the PA-phloroglucinol adduct (Brandon et al 1982). Aliquots of the reaction mixture (10 μl) were removed at 0-, 5-, 15-, and 30-min intervals and after 24 hr. These were examined by cellulose TLC using solvents A and B. The original unhydrolyzed material and authentic stands of (+)catechin and (−)epicatechin were chromatographed similarly. Hydrolysis products were identified by their mobility relative to authentic standards.

RESULTS

After hydrolysis of both ethyl acetate (EA1) and purified oligomeric (Aq2) fractions from bran by butanol/HCl, the absorption spectra of the hydrolysates showed anthocyanidins with maxima at 535 nm (ethyl acetate fraction) and 552 nm (oligomeric fraction), which suggested the presence of PAs. Measurement of anthocyanidins produced by hydrolysis of PAs in butanol/HCl has long been used as a means for detecting and estimating PAs in crude plant extracts (Porter et al 1986), and earlier attempts to detect PAs in crude extracts of wheat grain by this method yielded brown products with absorbance

maxima in the 450 nm region. A similar hydrolysis of purified methoxyhydroquinone (MHQ)-triglucoside in butanol/HCl yielded products with visible absorbance maxima also around 440–450 nm; we suggest, therefore, that earlier failures to detect PAs in crude wheat extracts in this and other studies (Pollock et al 1960) may have been due to interference by MHQ-glucosides, since these compounds are relatively abundant (150–180 μg/g, dw) in wheat grain (Daniels 1959, Bouvier and Horvath 1987). In this investigation, MHQ-glycosides and other impurities were removed by phase partitioning into water and dialysis during the prior preparation of bran fractions EA1 and Aq2; thus detection of PAs was made possible.

Hydrolysis of the ethyl acetate fraction yielded pelargonidin and cyanidin, plus a small amount of delphinidin. In contrast,
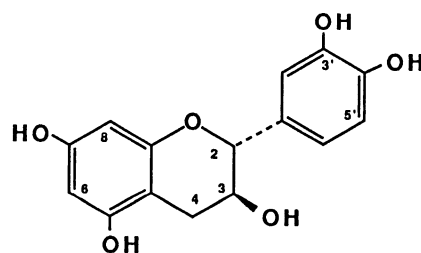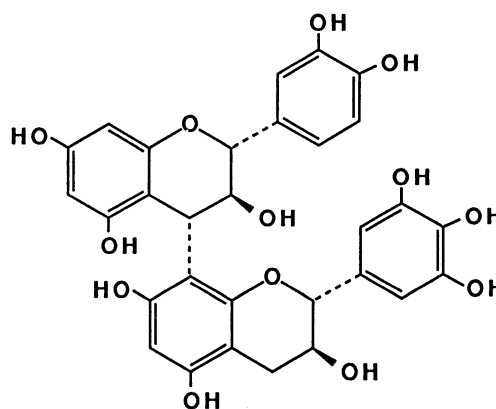

(+) Catechin
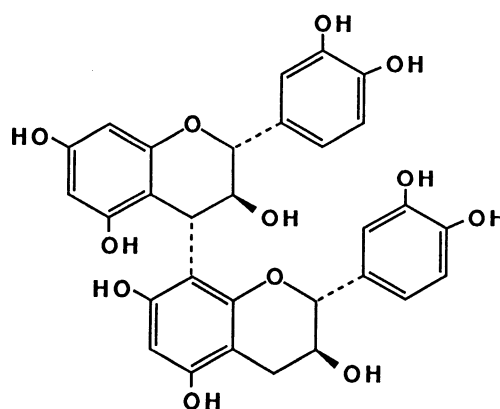
Procyanidin B-3

[catechin-(4α → 8)-catechin]

Prodelphinidin B-3

[gallocatechin-(4α → 8)–catechin]

Fig. 1. Structures of catechin and proanthocyanidins.

the oligomeric fraction yielded mostly delphinidin and a lesser amount of cyanidin. The identities of these anthocyanidins were confirmed by cochromatography with authentic standards and comparison of spectra with previously reported data (Jurd 1962). The PD/PCy ratio of the aqueous fraction was estimated to be approximately 70:30.

Cellulose TLC examination of the ethyl acetate fraction revealed the presence of at least three compounds whose chromatographic behavior and reaction with vanillin-HCl reagent was consistent with their being (+)catechin and PAs (Fig. 2). Other compounds were present also; these exhibited high retardation factors (R_f) in both TBA and 6% acetic acid and reacted with vanillin to give red and purple colors; these might be indoles or aromatic amines.

A similar TLC examination of the oligomeric fraction after preliminary purification on LH-20 showed the presence of one relatively discrete spot and a diffuse streak with little mobility in 6% acetic acid (Fig. 3). The chromatographic behavior suggested that these were a PA trimer and higher oligomers.

The PAs present in the ethyl acetate fraction were examined further by column chromatography on Sephadex LH-20 with ethanol as eluant. Fractions 6–11 (50–550 ml) contained a number of phenolics and two compounds that showed high mobility on cellulose and that reacted with vanillin to give dark purple and brown colors. Following the elution of most of the other phenolic material in the extract, a flavanol was eluted in fractions 15–19 that was identified as (+)catechin by cochromatography with an authentic sample in solvent systems A and B ($R_{fA} = 49$, $R_{fB} = 86$). This was followed by two other flavanols at much larger elution volumes, which suggested a more highly hydroxylated or polymerized state relative to catechin. These were obtained in sufficient quantity (approximately 2 mg each) by pooling fractions 36–42 (1,800–2,100 ml) (=PA1) and fractions 43–51 (2,150–2,550 ml) (=PA2) to permit partial characterization by means of acid hydrolysis in the presence of phloroglucinol. Traces of other vanillin-reactive flavonoids were detected by TLC but were not present in sufficient quantities to characterize.

Compound PA1 ($R_{fA} = 53$, $R_{fB} = 60$) yielded cyanidin on acid hydrolysis, and when this was performed in the presence of phloroglucinol, the products were identified by TLC as catechin and the catechin-phloroglucinol adduct ($R_{fA} = 60$, $R_{fB} = 57$). This suggested that PA1 was a dimeric procyanidin with the structure catechin-(4α→8)-catechin (Fig. 3), known by the trivial name of procyanidin B3 (Haslam 1982).

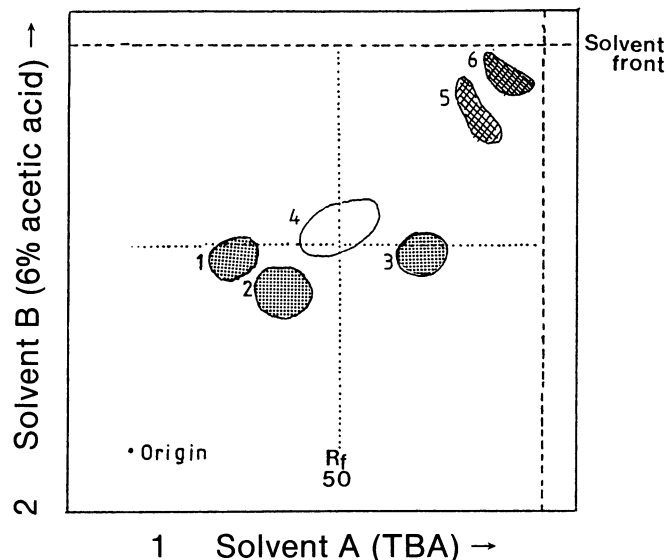Similarly compound PA2 ($R_{fA} = 42$, $R_{fB} = 57$) yielded delphinidin on acid hydrolysis and catechin plus the gallocatechin-phloroglucinol adduct ($R_{fA} = 35$, $R_{fB} = 64$) in the presence of phloroglucinol. This suggested that it was a dimeric prodelphinidin with the structure gallocatechin-(4α→8)-catechin (Fig. 3). This has the trivial name prodelphinidin B3 (McMurrough 1981).

Both of these dimeric PAs and (+)catechin have been reported as major components in the ethyl acetate fraction of barley phenolics (Brandon et al 1982), which also gave a similar value for the PD/PCy ratio in the oligomeric PA fraction. Failure to detect propelargonidins in the ethyl acetate extract was surprising in view of the relatively large yield of pelargonidin observed earlier after the hydrolysis of such extracts. They may have been due to losses or incomplete resolution from other phenolic material during chromatography. Propelargonidins have been reported from some European malting barleys but do not appear to be present in all barley cultivars (McMurrough 1981).

DISCUSSION

This study provided the first evidence that the bran from mature wheat grain contains (+)catechin and PAs. Although the amounts of extractable PAs in wheat appear to be significantly less than those reported for barley, our evidence suggests that these species are qualitatively very similar. Quantitative results were difficult to obtain, but on the basis of the observed yields and chromatographic evidence, it is estimated that the concentration of extractable soluble catechin plus PAs was in the order of 20–40 μg/g fresh weight bran.

Extraction of PAs from mature wheat grain is complicated by the presence of the various carbohydrate and protein fractions that readily complex with these compounds and also by the presence of active oxidative enzymes. Commonly only a small proportion of PAs can be extracted from plant tissue, and this may be as low as 10% in the case of polymeric prodelphinidins (Singleton 1972). Enzymic oxidation and complexation is favored by the breakdown of cellular structure that occurs in the seed coat during grain maturation (Percival 1921), and it may prove more informative to study the chemistry of PAs and other wheat grain flavonoids in the "green" seed prior to desiccation.

There is still a paucity of information concerning the chemical nature of the seed coat pigments of red wheats. Our observation that catechin and PAs are present in wheat bran supports the original hypothesis of Miyamoto and Everson (1958) that the red pigments may be formed by oxidation of these compounds,
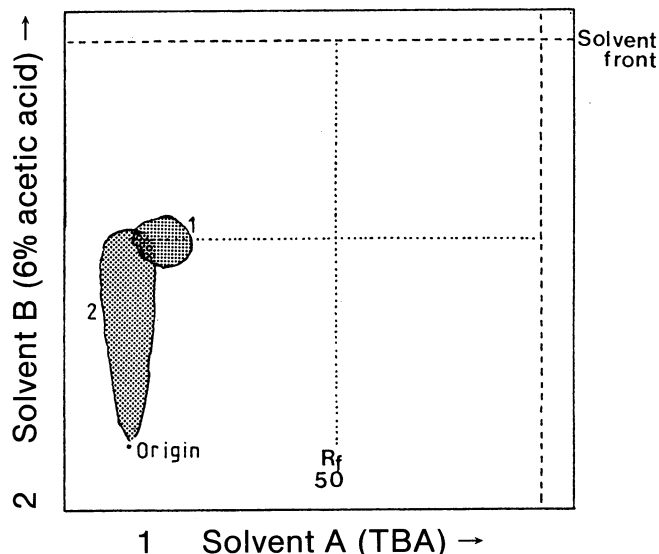

Fig. 2. Cellulose thin layer chromatography of ethyl acetate fraction (EA1): proanthocyanidin dimers (**1** and **2**); (+)catechin (**3**); C-glycosyl-flavone (visible under ultraviolet light) (**4**); unknown compounds reacting with vanillin/HCl (**5** and **6**).

Fig. 3. Cellulose thin layer chromatography of oligomeric fraction (Aq2): proanthcyanidin trimer (**1**); higher molecular weight proanthocyanidin oligomers (**2**).

and in the presence of amino acids and crude wheat grain enzyme extracts, small amounts of catechin will yield red pigments. Further evidence supporting this hypothesis comes from recent work on the nature of kiwifruit skin pigmentation (D. Rowan and G. Lane, *personal communication*). These workers observed that PAs isolated from kiwifruit skin showed a pH-sensitive color change after oxidation but not before. They suggested that the darkening of kiwifruit skin at high pH may be analogous to the characteristic brick-red colors exhibited by red wheat seed coats after steeping in alkali. In both cases the color changes may result from the indicator properties of oxidized PAs.

PAs may also contribute to the color of bran and wheaten products through several other mechanisms (Singleton 1972). Complexation of PAs with ferric iron or copper produces strongly colored complexes. Under weakly acidic conditions formation of xanthylium salts in situ may occur in PAs, and this may also contribute to the color of these compounds. Furfural derivatives formed during the caramelization and breakdown of sugars (notably from pentosans in wheat flour) may undergo condensation with PAs during baking to produce colored products employing reactions analogous to the vanillin reaction used to detect PAs.

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