965

soy peroxidase to evaluate these observations.

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High-Performance Liquid Chromatography of Procyanidins in Developing Sorghum Grain

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The development of low molecular weight procyanidins was studied in bird-resistant sorghum grain during the reproductive developmental stages. It is the procyanidins which inhibit the amylase enzymes which are required during the brewing of sorghum beer. Grains were collected at various stages of maturity, and their procyanidins were monitored by using high-performance liquid chromatography (HPLC) on silica gel with anhydrous organic solvents. Sufficient material for chemical analysis was obtained from column chromatography of acetylated plant extracts. The melting point depression of camphor was used for molecular weight determination of the acetylated procyanidins. During the flowering stage, catechin was detected, but as the grain matured, several low molecular weight procyanidins were present. They reached a maximum concentration at the soft dough stage. As the grain matured, the low molecular weight procyanidins could no longer be detected, indicating the end of the period of their synthesis. By the melting point depression of camphor, one dimer and two trimer isomers of procyanidin were identified in immature sorghum grain.

Procyanidins, or condensed tannins, occur in the testa of bird-resistant sorghum grain [*Sorghum bicolor* (L.) Moench] (Rooney et al., 1979; Morrall et al., 1981). The chemical nature of procyanidins makes them difficult to

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isolate and purify. Although it is believed that simple mono-, di-, and trimeric forms are the basis for the synthesis of these high molecular weight polymers, they are not found in mature sorghum grain.

Amylase enzymes were found to be inhibited by the sorghum grain procyanidins (Strumeyer and Malin, 1969), and these enzymes are required during the brewing of sorghum beer (Daiber, 1975). When amylase inhibition by sorghum grain was studied over the 10-week preharvest
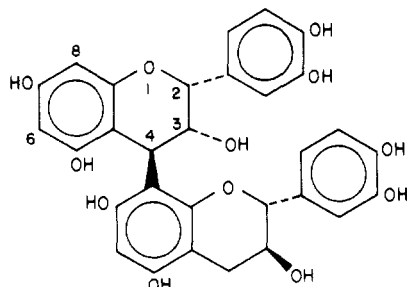
Figure 1. Structure of dimeric procyanidin.

period, it was found that enzyme inhibition increased until ~6 weeks before harvest. After this peak, the inhibition declined until the time of harvest, but inhibitory activity was still present (Davis and Hoseney, 1979). In another study where the procyanidins were extracted and assayed with a modified vanillin method, a similar pattern of preharvest procyanidin content was observed (Price et al., 1979).

Other than these patterns of change in the extractable procyanidins in sorghum grain, little is known about their synthesis and deposition. Gupta and Haslam (1978) suggested that the polymeric procyanidins could be formed from (+)-catechin and an epicatechin carbocation to form a dimer (Figure 1) and then condense with more carbocations of the same structure to form a polymer. They also suggested that the monomer and dimer appear at the stage when chlorophyll develops in the pericarp and that these forms disappear as the seed matures. At maturity, only high molecular weight procyanidins could be found.

The procyanidins of cider were successfully separated by HPLC using various reverse-phase column packings and acidified water–methanol eluants (Lea, 1979, 1980). Also, hydrolyzable tannins were separated by HPLC using a silica gel (Corasil II) packing with an organic solvent gradient elution (Beasley et al., 1977).

With HPLC techniques making possible the separation of the various molecular weight forms of procyanidins, it became possible to study their development in immature sorghum grain. A profile of procyanidin development could be built up by subjecting the extracts of the preharvest grain to HPLC separation. This paper reports the development and disappearance of low molecular weight procyanidins in sorghum grain.

MATERIALS AND METHODS

Sorghum grain was collected at various preharvest stages, and this study was carried out to determine the pattern of procyanidins as the grain matured. Since it was not possible to separate the various molecular weight forms of procyanidin by paper chromatography, other separation techniques had to be employed. Gel filtration chromatography offered a certain resolution of procyanidins. Using Sephadex LH-20, Strumeyer and Malin (1975) were able to separate sorghum procyanidins into several fractions. Two other supports, Sepharose Cl-6B and Sephacryl S-200, also were able to fractionate sorghum procyanidins (Kaluza et al., 1980). While these methods offered some resolution, they are time consuming so separation by HPLC was examined.

Bird-resistant sorghum, hybrid SSK 52, was grown at the Plant and Seed Control Division, Roodeplaat Experimental Farm, South African Department of Agriculture and Fisheries. The row spacing was 0.8 m while the plant spacing within the rows was 8 cm. The seed was planted on Oct 9, 1979, with emergence occurring on Oct 16, and the first collection at the preflowering stage was taken on Jan 3, 1980. Thereafter, samples were collected at 5-day

intervals except for a 48-day interval between physiological maturity and time of harvest. Ten heads were collected at each stage and the collected grain was hand threshed and freeze-dried. The growth stages of the grain agreed with those described by Vanderlip and Reeves (1972) except that the grain in this study matured quicker.

The dried grain was ground for 1 min in a Janke and Kunkel mill (a water-cooled coffee mill) and extracted with 70% aqueous acetone on a Buchner funnel by using 500 mL of 70% acetone/100 g of ground grain. The extract from the soft dough stage was concentrated in a rotary evaporator and streaked on Whatman 3MM paper for descending chromatography in 1-butanol–acetic acid–water (BAW), 4:1:5, top layer. After chromatography, the procyanidin-containing bands were located by dipping a strip of the chromatogram into vanillin reagent and heating at 80 °C (Gupta and Haslam, 1978).

The positive bands were eluted and taken to dryness in a rotary evaporator. The residue was acetylated in acetic anhydride and pyridine at room temperature overnight. The acetylated residue was subjected to column chromatography on silica gel (Merck Kieselgel 60) using benzene–acetone (4:1) as the solvent. The elution pattern of the column was followed by spotting the fractions on precoated fluorescent silica gel TLC plates (Merck) and developing them in the column solvent. The spots were located under UV light.

The determination of molecular weights was carried out by Rast's camphor method as described by Vogel (1948). A small sample of acetylated compound was melted with 10 times its weight of pure resublimed camphor (BDH), and its melting point determined. The melting point of the resublimed camphor was determined, and the depression of the melting point caused by the acetylated procyanidin was used to calculate its molecular weight.

A small amount of the 70% acetone extract was banded on precoated TLC plates (Merck Cellulose F) and run in BAW. This yielded small amounts of the various procyanidins. The correct bands were located by spraying a small strip along the edge of the plate with vanillin reagent. These bands were removed and eluted. Aliquots of the individual bands were subjected to HPLC while aliquots were acetylated and chromatographed on silica gel.

HPLC was carried out on aliquots of this extract from each of the different stages of development. The chromatograph used was a Varian Model 5000 with an LDC UV III monitor with a 280-nm filter. The column (25 × 0.4 cm) was packed with LiChrosorb Si 60 (5 μm). The solvent which gave the best separation was prepared from 200 mL of anhydrous tetrahydrofuran and 600 mL of anhydrous methanol with 8 mL of glacial acetic acid (A) or 40 mL solvent A diluted to 1 L with anhydrous hexane (B) (Beasley et al., 1977). The solvent was run isocratically at 48% B (52% A) at 1 mL min^{-1}. The capacity factor was calculated from the retention times of the different procyanidins (t_R) and the retention time of a nonretaining peak (t_0) according to

$$k' = (t_R - t_0)/t_0$$

RESULTS AND DISCUSSION

The term procyanidin is used here as cyanidin was the only anthocyanidin released on heating in dilute acid. Also, it is probable that the low molecular weight forms of the procyanidins would not be effective tanning agents and are, therefore, not true tannins.

Separation of sorghum procyanidins by paper chromatography showed poor resolution. Chromatography in BAW yielded three vanillin-sensitive bands: a broad band

LC of Procyanidins in Developing Sorghum Grain

J. Agric. Food Chem., Vol. 29, No. 5, 1981 **967**

Table I. R_f Values, HPLC Capacity Factors, and Molecular Weights of Some Procyanidins Isolated from Sorghum Grain

compound	R_f(TLC)[a] in BAW	R_f(TLC)[b] in benzene–acetone	HPLC capacity factor (k')	obsd M_r	calcd M_r
catechin std	79	58	0.46	555	500
monomer	79	59	0.46	476	500
dimer	70	43	1.15	904	998
trimer a	56	36	1.77	1583	1496
trimer b	44	33	2.54	1488	1496
unknown a	37	26	3.15		
unknown b	29	13	6.23		
unknown c			10.08		

[a] R_f (×100) of procyanidins on cellulose. [b] R_f (×100) of acetylated procyanidins on silica gel.

between R_f 0.30 and R_f 0.70 and two narrow bands of R_f 0.16 and 0.05, respectively. The broad band was colorless while the two narrow bands were a light brown in color as was the origin. All three bands and the origin developed the red color typical of procyanidins when reacted with vanillin.

Column chromatography was used to obtain sufficiently large amounts of the procyanidins for chemical analysis. The bands from paper chromatography of soft dough extracts were acetylated and chromatographed on columns of silica gel. The broad band from 3 MM paper yielded six acetylated procyanidins in various amounts, presumably as they occurred in the grain. The compounds were further chromatographed on the column until they were obtained as white powders. These powders could not be crystallized but appeared to be chromatographically pure. Table I shows that this method was only successful in separating the low molecular weight procyanidins. Higher molecular weight procyanidins, such as the other two vanillin-positive bands from the paper, did not move from the origin when acetylated and chromatographed on silica gel.

TLC on precoated cellulose plates using BAW as the solvent resolved the procyanidins into many more bands than did the paper. R_f values are presented in Table I. Preparative TLC plates did not give the resolution of the precoated plates so that large amounts of clean procyanidin of a single molecular weight could not easily be obtained. The small samples isolated from TLC were used to coordinate the acetylated fractions from the silica gel column with the peaks from HPLC.

The properties of the sorghum procyanidins were characterized by using several methods. When viewed under UV light, all compounds adsorbed and were easily located on fluorescent TLC plates. The seven fractions separated by HPLC were collected individually and their UV spectra determined in 80% ethanol. The spectra of all seven procyanidins were identical, giving a peak at 274 nm as did commercial catechin. Hydrolysis with boiling 2 M HCl yielded cyanidin as the only anthocyanidin. It was identified by direct comparison with authentic cyanidin by color, R_f values, and UV spectra.

Mass spectra did not show molecular ions for any acetylated procyanidin larger than the monomer which gave a molecular ion at 500 as calculated. Since mass spectrometry failed to give the molecular weights of the larger procyanidins, more traditional methods of molecular weight determination were examined. The Rast method, which depends on the melting point depression of camphor, yielded good results. These are given in Table I. This identified the first two peaks from HPLC as the monomer and dimer, respectively, while the following two peaks were apparently isomers of trimeric procyanidins. Even though the differences in structures between the two isomers are unknown, the complexity of the procyanidin patterns in sorghum grain is confirmed.
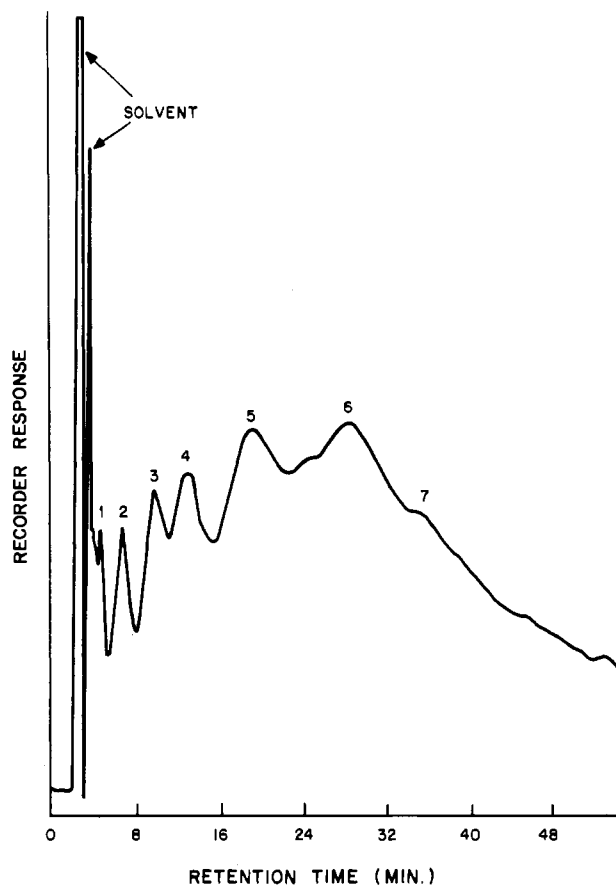

Figure 2. HPLC of procyanidins in soft dough stage of sorghum grain on LiChrosorb Si 60 (5 μm) with solvents A:B (52:48) (A = tetrahydrofuran–methanol–acetic acid, 200:600:8; B = hexane–A, 96:4). The flow rate was 1 mL min⁻¹, and a UV detector with a 280-nm filter was used at a sensitivity of 0.064. 50 μL was injected.

The finding of two trimeric isomers indicates that the higher molecular weight procyanidins found in sorghum grain could be branch-chained structures. The trimeric isomers contain nucleophilic centers at positions 6 and 8 (see the dimer, Figure 1) and can act as key intermediates in procyanidin synthesis (Roux et al., 1980). The number of available nucleophilic centers probably favors procyanidins of a complex structure.

The low molecular weight procyanidins were separated successfully by HPLC. This technique has the advantage of being more rapid than the others as well as giving highly reproducible results. Because of this, HPLC was chosen to monitor the changes of procyanidins in the maturing sorghum grain. The capacity factor (k') for the different procyanidins is given in Table I. HPLC was used to estimate similar phenolic compounds found in malts and beers (Jerumanis, 1979).

The separation of sorghum procyanidins by HPLC is shown in Figure 2. This extract was from the soft dough

Table II. Peak Heights of Different Procyanidin Polymers Isolated by HPLC[a]

stage of maturity	peak heights, mm, for peak no.[b]						
	1	2	3	4	5	6	7
preflower	>215	0	0	0	0	0	0
midflower	215	0	0	0	0	0	0
late flower	192	0	0	0	0	0	0
milk	163	54	42	51	44	42	0
soft dough	180	46	54	71	68	66	63
hard dough	35	33	39	52	54	49	52
physiologically mature	81	0	35	35	30	48	58
time of harvest	79	0	0	0	41	54	53

[a] 50 μL was injected from a 10 mg of ground grain/mL of 70% acetone extract. [b] 1 = catechin; 2 = dimer; 3 = trimer a; 4 = trimer b; 5 = unknown a; 6 = unknown b; 7 = unknown c.

stage which contained the full complement of low molecular weight procyanidins. The more immature stages did not contain all the peaks shown here, and in the more mature stages the peaks of the lower molecular weight compounds began to diminish and disappear. Two properties were observed which seemed to be characteristic of procyanidins when run on HPLC. The pen did not return to base line between peaks, and this appeared to be independent of the solvents and column packings used. Also, the peaks became broader as the molecular weights of the procyanidins increased. The peak broadness could be due to a mixture of isomers eluting together as one peak; i.e., the chromatographic system failed to separate them.

The patterns of the procyanidins for all developmental stages were incorporated into Table II. The peak heights are reported, and they show the development of the low molecular weight procyanidins in the immature sorghum grain. Catechin was the only procyanidin compound present during the flowering stage. Only after fertilization occurred and the inner integument developed into a testa did the rate of procyanidin concentration increase. During the milk, soft dough, and hard dough stages, the catechin content decreased. Presumably this represents a period of rapid procyanidin synthesis when the catechin was being

incorporated as the terminal unit of the polymer.

The bulk of procyanidin synthesis must have occurred during this period as in later stages of grain development neither the dimer nor trimer forms could be found. The mature grain contained only the high molecular weight forms of procyanidin. This pattern agrees with other work (Glennie, 1981) where extractable procyanidins were measured and found to reach a maximum at the hard dough stage. The HPLC patterns are consistent with the idea that procyanidins develop sequentially from a monomer through dimer and trimers, until they reach high molecular weight forms which are probably insoluble.

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Rye Prolamins: Extractability, Separation, and Characterization

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Compared with some concentrations of aqueous 1-propanol and 2-propanol, 60% (w/w) ethanol was found to be the best extractant of rye prolamins (secalins) with respect to purity. Rye meal defatting with water-saturated 1-butanol before extraction resulted in a 40% loss of secalin yield. Secalins were separated by ion-exchange chromatography and gel filtration. They are distributed in three groups named A, B, and C by order of increasing molecular weight (M_r). Like ω-gliadins and C hordeins, C secalins (M_r 38 000) have high Glx and Pro contents and are free of sulfur amino acids. B secalins (M_r 29 000) are similar to α-, β-, and γ-gliadins and therefore are probably the only secalins noxious for gluten-sensitive people. A secalins (M_r 16 000) have molecular weights in the same range as low molecular weight gliadin and A hordein molecular weight. They are Lys and His free and have much higher sulfur amino acid and Tyr contents than other secalins. Leu, Asx, and Arg are the N-terminal amino acids of A, B, and C secalins, respectively.

Like wheat, rye can be used for bread making though its baking quality is clearly lower than that of wheat.

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Moreover, rye is noxious for gluten-sensitive people although to a lesser extent than wheat (Dicke et al., 1953; Anand et al., 1978; Kasarda, 1978). Therefore rye is an interesting material for the study of relations between protein composition and functional properties on the one hand and for the study of relations between protein com-