AN EXAMINATION OF ANTHOCYANOGENS IN GRAIN SORGHUMS[1]

C. W. BLESSIN, C. H. VANETTEN, AND R. J. DIMLER

ABSTRACT

Anthocyanogens (leucoanthocyanins) were detected in yellow milo and red kafir sorghums but not in white kafir, waxy, and yellow-endosperm varieties. When present, these compounds are located mainly in the pericarp and are generally absent from the endosperm. The anthocyanogens in aqueous extracts of the whole grain were isolated by a procedure based on adsorption by strongly basic ion exchange resins. Spectral data on the purified material in neutral, acidic, and basic solvents are discussed. The usual methods for converting anthocyanogens to anthocyanidins were not applicable. Fisetinidin was tentatively identified as one of the reaction products resulting from treatment of the anthocyanogens with 12*N* hydrochloric acid at room temperature. Spectral data on the purified material at various oxidation stages are presented.

Sorghum grain contains varying amounts and types of pigments in the pericarp and nucellar layer (7). Some of these pigments may migrate into the endosperm during the steeping operation for wet-milling or during tempering for dry-milling. Starch from wet-milling and grits from dry-milling may therefore contain objectionable pigments.

A preliminary examination of the water-soluble pigments in several domestic varieties of grain sorghum indicated the presence of leucoanthocyanins.[2] The general term "anthocyanogens" proposed (5) for these compounds is used in this paper. This group of pigment precursors was selected for further examination since they may be responsible for the development of a number of related pigment types (16) during processing of grain sorghum products. In addition, the anthocyanogens apparently impart astringency to foods and beverages (2). The bitter flavor of brown-seeded grain sorghum,[3] which has been ascribed to tannins, may result in part from anthocyanogens. Several investigations have indicated that these polyphenolic compounds are precursors of condensed tannins (13,14).

This report describes the location, extraction, purification, and a study of general properties of anthocyanogens in yellow milo and red kafir grain sorghums.

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[2]Blessin, C. W., Dimler, R. J., and Senti, F. R. Water-soluble pigments of sorghum. Sorghum Newsletter (Kansas Agr. Exp. Sta., Hays, Kans.), vol. 2, p. 19 (1959).

[3]Brown, A. R. Sorghum Newsletter (Kansas Agr. Exp. Sta., Hays, Kans.), vol. 2, p. 13 (1959).

Materials and Methods

Materials. Domestic varieties of yellow milo and kafir sorghums and of sorghums from Africa were investigated. Certified seed samples were used for large-scale preparations to eliminate contamination.

Location of Anthocyanogens. Examination of the seed and pericarp of Martin sorghum, a typical yellow milo variety, cut longitudinally, revealed five major areas of pigmentation — the dark-brown seedtip; the orange pericarp; the light-yellow embryo; the yellow, yellow-orange, or purple corneous endosperm; and the white starchy endosperm. Treatment with 12*N* hydrochloric acid developed a magenta color in the pericarp and seedtip, as well as in the corneous endosperm of the occasional kernels having purple color in this area. No color change was noted in either the embryo or the starchy endosperm. Treatment with aqueous 0.1*N* sodium hydroxide developed a yellow color in the pericarp and embryo and either a red-orange or purple color in the seedtip. These qualitative tests indicated that the compound or compounds with anthocyanogen properties are located in the pericarp and seedtip and are essentially absent from the endosperm.

Placing the whole sorghum grain in aqueous 12*N* hydrochloric acid at room temperature provided a rapid method for detecting anthocyanogens. The magenta color, which first develops in the pericarp, was extracted into the aqueous hydrochloric acid solution. Slight amounts of heat, accompanied by shaking, accelerated the test.

Examination of several foreign and domestic grain sorghums with variously colored pericarps indicated that pericarp color is not related to anthocyanogen content. Domestic varieties of yellow milo (Martin, Midland, and Westland) and red kafir contained considerable amounts of these anthocyanogens. Little or none was detected in white kafir, waxy, and yellow endosperm varieties, as well as in some highly pigmented sorghums (Pictoria Pink, Kershoma Purple, and Pink Kafir). Absence of anthocyanogens in certain varieties of kafir corn (grain sorghum) was reported in malting studies on these grains (10).

Preparation of Resin. One hundred and fifty grams of wet Dowex 1 × 10,[4] chloride form, 50- to 100-mesh, were treated with 200 ml. aqueous 2*N* sodium hydroxide by a batch-slurry method. The alkaline solution was decanted and then the resin was washed to pH 7 with distilled water. The resin was then slurried with 200 ml. aqueous 6*N* hydrochloric acid followed by washing to pH 5 with distilled water.

[4] The mention of trade products does not imply that they are endorsed or recommended by the Department of Agriculture over similar products not mentioned.

Two hundred milliliters of a saturated solution of sodium acetate in water converted the chloride form of the resin to the acetate form. A column was packed with the resin and then thoroughly washed with distilled water to remove sodium acetate. The resin was successively extracted with 200 ml. of methanol, 200 ml. of 33% glacial acetic acid-67% methanol (by weight), and 200 ml. of 67% glacial acetic acid-33% methanol (by weight). These solvents remove considerable amounts of ultraviolet-absorbing material which interferes with spectral measurements in the 280-mμ region. The column was then thoroughly washed with water. Resin used for the isolation of anthocyanogens was regenerated by this procedure.

Extraction and Purification of Anthocyanogens. Whole Martin sorghum grain was cleaned in a laboratory air cleaner to remove glumes and other foreign matter. One kilogram of the cleaned grain was extracted twice with diethyl ether (peroxide-free) at room temperature to remove the external wax coating. The grain was dried and extracted twice for 10 minutes on a steam bath with 1,250-ml. portions of water.

Two hundred and fifty milliliters of glacial acetic acid were added to the extract to ensure mildly acid conditions. The solution was extracted with three 1,250-ml. portions of diethyl ether. Tests for anthocyanogens in the yellow diethyl ether extracts were negative. The aqueous acid phase was extracted with n-butanol using 1,250 ml. for the first and 625 ml. each for the second and third extractions. The n-butanol extracts were combined and concentrated to dryness under vacuum. The yellow-brown aqueous phase was discarded. The dry solid was extracted with water adjusted to pH 5 with glacial acetic acid.

An aliquot of the soluble fraction was put on a column of prepared Dowex 1 $\times$ 10 resin, 12 $\times$ 600 mm., to the capacity of the resin for the anthocyanogens. Resin capacity was determined by the method described below. The column developed a dark red-brown color. After washing with 200 ml. water, residual water was displaced and the column conditioned with 100 ml. methanol. Sufficient 67% glacial acetic acid-33% methanol (by weight) then was passed through the column to change the color of the resin from red-brown to yellow; this treatment ensured removal of materials soluble in methanol. The anthocyanogens were then eluted with 200 ml. 67% glacial acetic acid-33% methanol. The column was reconditioned by washing with 100 ml. water.

The formation of a stable magenta color on treatment of anthocyanogens with aqueous 12*N* hydrochloric acid at room temperature was used to determine the capacity of the resin and to measure the re-

covery of anthocyanogens on elution from the column. One milliliter of the effluent and 9 ml. 12*N* hydrochloric acid were reacted for 10 minutes. The absorbance was measured at 548 mμ. A similar determination was made on the crude water extract put on the column. Although absolute values for anthocyanogen content could not be calculated, excellent reproducibility and recoveries were obtained in determining relative amounts of anthocyanogens in effluents from the column and in extracts from sorghum grains.

The column effluents containing the anthocyanogens were combined and concentrated to dryness under vacuum. The dry residue was a brown hygroscopic solid with a phenolic odor. This purified anthocyanogen material exhibited limited solubility in water, moderate solubility in alcohols, and complete solubility in acids and bases. Phosphorus and nitrogen analyses were negative. Microanalysis on a humidified and redried sample gave carbon 55.4%, hydrogen 6.0%, and ash 6.0%.

Paper Chromatography. The hot water extract of whole grain and the purified anthocyanogens were chromatographed on Whatman No. 1 paper with water-saturated secondary butanol as the developing solvent in direction 1 and with 2% glacial acetic acid in water for direction 2. The papers were equilibrated for 8 hours with water-saturated secondary butanol prior to 30-hour development with that solvent and for 1 hour with 2% acetic acid in water prior to 8-hour development in direction 2 (13). Fluorescent materials were detected by long-wave ultraviolet light (360 mμ) in the presence of ammonia to enhance fluorescence. Anthocyanogens or "leuco" compounds were determined with *p*-toluenesulfonic acid spray (13).

Anthocyanidins were chromatographed on Whatman No. 1 paper using 90% (w/v) formic acid-3*N* hydrochloric acid (1:1, v/v) in direction 1 and water-glacial acetic acid-12*N* hydrochloric acid (10:30:3, v/v) in direction 2 (12). After calculation of the R_f values of the scarlet spots, excess hydrochloric acid was removed by airing at room temperature. Treatment of the anthocyanidins (now colorless) with ammonia produced a distinct blue.

Results and Discussion

Extraction of anthocyanogens from whole grain, ground whole grain, and ground pericarp tissue by various solvents was investigated. No single solvent system was ideal for removing anthocyanogens. Hydrocarbons, ketones, and ethers were ineffective. Both methanol and ethanol extracted magenta pigments (tentatively identified as anthocy-

anins or anthocyanidins) with the anthocyanogens. Mineral acids in water or alcoholic solvents were avoided, since anthocyanogens in grain sorghums are sensitive to those solvents. Water was selected as the solvent to preserve the compounds as unaltered as possible, although it had the disadvantage of extracting considerable carbohydrate material. Extraction of either intact whole grain or separated ground pericarp with water was more effective than extraction of ground whole grain. Considerable adsorption of the extracted anthocyanogens apparently occurred on the endosperm fragments in the ground grain. MacMasters and Hilbert (7) showed that water-soluble pigments from certain sorghum varieties were strongly adsorbed on starch during processing.

Because ion exchange resins have been used to separate flavonoids in apricots from their associated sugars (18), trials of purification of the anthocyanogens in grain sorghums were made with four general types — strongly acidic, weakly acidic, strongly basic, and weakly basic. Dowex 1, a strongly basic resin, was the only one among the resins tried that gave complete retention of the anthocyanogens.

Elution from Dowex 1 × 1 in different salt forms (acetate, oxalate, and sulfite) by various solvents was investigated. Although aqueous solutions of acids and salts were poor eluting agents, alcoholic solutions were fair to excellent. A saturated solution of oxalic acid in methanol gave complete elution. Disadvantages associated with using oxalic acid as an eluting agent include reaction with anthocyanogens upon removal of methanol (residual crystals of oxalic acid are pink) and the difficulty of obtaining the anthocyanogen preparation devoid of oxalic acid. Alcoholic solutions of strong mineral acids were ineffective. Methanolic solutions of glacial acetic acid were fair eluting agents. An increase in the glacial acetic acid concentration from 33 to 66% by weight increased the recovery of the anthocyanogens put on the column from 26 to 66%. Acetic acid did not react with the anthocyanogens under these conditions and was volatile enough to be removed with relative ease.

Treatment of the resin with 12*N* hydrochloric acid following elution with 66% glacial acetic acid in methanol gave a red color, which indicated retention of some anthocyanogens in the resin. The red pigment was not eluted from the resin by further volumes of 12*N* hydrochloric acid. The types of eluting agents (combination of organic acid and organic solvent) that were effective and the retention of part of the anthocyanogen on the resin indicate that initial retention of the material on Dowex 1 is to a large degree the result of adsorption in or on the resin, although the simultaneous function of ion exchange is

not excluded. Such adsorption on the organic resin would be analogous to the adsorption of anthocyanogens of beer on nylon, described by Harris and Ricketts (6).

The effect of degree of cross-linking of Dowex 1 was investigated to determine if any specific degree of cross-linking was advantageous to the separation of the anthocyanogens. Although the material was retained by all the resins, ranging in cross-linkage from 1 to 10, it was eluted more readily from Dowex 1 × 10 than from the other resins.

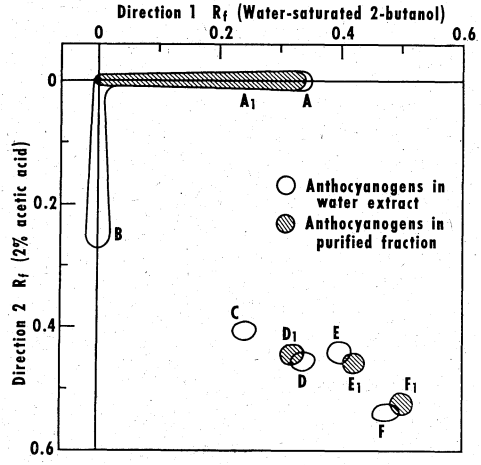

Fig. 1. Two-dimensional chromatogram of sorghum anthocyanogens.

A two-dimensional chromatogram (Fig. 1) of the hot water extract of whole grain, sprayed with *p*-toluenesulfonic acid reagent, developed six areas that contained anthocyanogens together with considerable fluorescent material. (Fluorescent compounds are not indicated on the chromatogram.) The immobile anthocyanogens (A and B) remaining at the origins are apparently polymeric materials (13). The four distinct anthocyanogens (C, D, E, and F) have R_f values similar to those reported for monomeric and dimeric tri- and tetra-hydroxyflavan-3,4-diols (13,15).

Purification on Dowex 1 × 10 resin eliminated most of the fluorescent material and concentrated the major portion of the anthocyanogens into one fraction (E_1 in Fig. 1) having an R_f of 0.44/0.47 (Fig. 1). Although minor amounts of polymeric material (A_1) and other anthocyanogens (D_1, F_1) remained in the purified fraction, the material was considered "pure" enough for further investigations.

Absorption data (Fig. 2) obtained on the Cary recording spectro-

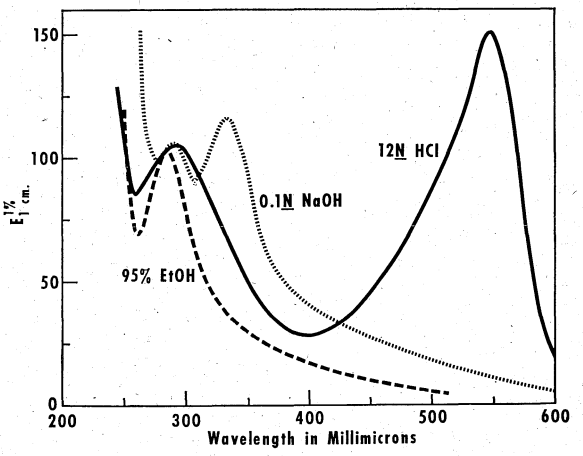

Fig. 2. Visible and ultraviolet spectra of purified sorghum anthocyanogens.

photometer are reported in the form of $E^{1\%}_{1\ cm.}$ values since the molecular structure is uncertain. The absorption maximum near 280 mμ for the purified material in 95% ethanol solution is typical of polyhydroxyphenols that contain no carbonyl conjugation (4). The absorption spectrum in aqueous 0.1*N* sodium hydroxide indicates retention of the polyhydroxyphenol character (280 mμ), with development of a maximum at 330 mμ indicative of carbonyl conjugation. However, at the present stage of purification a part of the 330-mμ absorption may be due to the presence of flavanone-type compounds, which give a shift in the absorption spectrum upon treatment with alkali. Treatment with 12*N* hydrochloric acid at room temperature did not alter the ultraviolet absorption characteristics (no change in $E^{1\%}_{1\ cm.}$ at 280 mμ). The development of the magenta color with its absorption maximum at 548 mμ denotes the formation of an oxonium-type structure. Carbon and hydrogen values are similar to those reported for cacao anthocyanogens (3). A negative nitrogen test indicated the material is not a pyrrole derivative or "nitrogenous" anthocyanogen (8). Physical and chemical data indicate that the material is of the leucoanthocyanin type having a flavane structure. The rapidity of reaction with cold hydrochloric acid indicates some of the material is in the pseudobase form (17).

Bate-Smith's (1) and Pigman's (9) methods, normally used for the generation of anthocyanidins, failed to produce a stable magenta coloration when applied to the purified material isolated from grain sorghum. However, cold (room-temperature) 12*N* hydrochloric acid produced a magenta pigment with a spectrum typical of anthocyani-

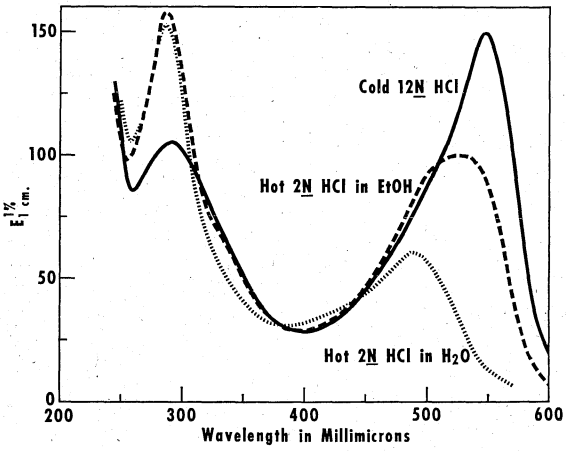

Fig. 3. Absorption spectra of purified sorghum anthocyanogens at various oxidation stages.

dins (Fig. 3). Refluxing for 60 minutes with 2*N* hydrochloric acid in absolute ethanol produced an orange-magenta solution with reduced absorption in the visible range. Refluxing 30 minutes with aqueous 2*N* hydrochloric produced an orange solution retaining no anthocyanidin characteristics. The greater absorption at 280 mμ of the 2*N* hydrochloric acid reaction products may be due to acid degradation fragments. Although a magenta pigment was initially produced in the alcoholic and aqueous 2*N* hydrochloric acid reaction mixtures, a rapid change to orange-red occurred on further heating. Chromatography of the orange-red or orange solutions with the two solvent systems gave no evidence of anthocyanidins. Treatment of the original orange-red or orange solutions with magnesium ribbon plus 12*N* hydrochloric acid produced a red solution; this transformation is characteristic of flavonols, flavonones, flavonals, or flavones. Apparently the magenta anthocyanidin-type material had been converted by oxidation to an orange flavonol-type product upon further heating (11). This theory is in agreement with Roux and Evelyn (14) who found that robinetinidin and fisetinidin undergo partial oxidation into the corresponding flavonols during their generation from anthocyanogens.

Treatment of the purified material with 12*N* hydrochloric acid at room temperature developed a magenta color, which was stable for at least 24 hours. Chromatography of the n-butanol extract of this solution indicated the presence of only one anthocyanidin (R_f 0.51/0.77) along with considerable orange-colored materials. Chromatography of a hot absolute ethanol extract of the whole grain also indicated the

presence of small amounts of a free anthocyanidin (R_f 0.45/0.73) in the pericarp. These values agree fairly well with the R_f values for fisetinidin (R_f 0.43/0.77) reported by Roux (12).

Differences in the absorption maxima of 525 mμ reported for fisetinidin (12) compared to 548 mμ for the isolated anthocyanogen material indicated the presence of additional anthocyanidins in the acid solution. Failure to detect other anthocyanidins may be due to their rapid conversion to flavonol-type compounds. Another possible explanation is the formation of related anthocyanogens and polymeric materials by the interconversion of several flavan-3,4-diols (16). These observations indicate that the purified anthocyanogen fraction isolated from grain sorghum may possess more structural complexity than is suggested by preliminary results based on paper chromatography.

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