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Preharvest Changes in Polyphenols, Peroxidase, and Polyphenol Oxidase in Sorghum Grain

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At various stages of preharvest development, "bird-resistant" sorghum [*Sorghum bicolor* (L.) Moench] grain was analyzed for tannin content and polyphenol oxidizing enzymes. Tannins appeared first at the milk stage, reached a maximum at the hard dough stage, and thereafter declined by 25%. Polyphenol oxidase (PPO) and peroxidase activities were detected during the flowering stage. PPO activity declined rapidly as the grain began to develop while the decline of peroxidase was slower. Neither activity could be detected in mature grain. Both enzymes were characterized and PPO behaved as a catechol oxidase. It appeared that these enzymes were not responsible for tannin deposition in sorghum grain. The decrease in extractable tannin was attributed to the high molecular weight tannin complexing with cellular components to become insoluble.

Sorghum grain polyphenols, in the form of condensed tannins, protect the grain against depredation by birds but can reduce the nutritional quality of the grain (Chavan et al., 1979). In southern Africa where the grain is used for brewing sorghum beer, the tannins inhibit enzymes required during brewing (Daiber, 1975).

Davis and Hoseney (1979) determined the preharvest changes in the tannin content of bird-resistant sorghum grain using its ability to inhibit α-amylase. They found that enzyme inhibition increased gradually until ~6 weeks before harvest. After that, inhibition levels decreased until the time of harvest. In another study, in which tannin was measured by a modified vanillin assay, a similar pattern was found (Price et al., 1979). The amount of extractable tannin increased to a maximum and then decreased. There was wide variation in the decline of tannin in the nine different cultivars studied.

A similar pattern occurs in ripening fruit (bananas, plums, and peaches) where there is a loss of astringency as the fruit ripens. Goldstein and Swain (1963) suggested that this loss of astringency was probably connected with the polymerization of the tannins to increased molecular weight. Two theories which account for tannin polymerization involve either an enzymatic or a nonenzymatic process (Brown, 1964). Polyphenol oxidases were associated with the aerobic oxidation of gallocatechin to form tannins in the cambrium layer of oak trees (Hathway and Seakins, 1957; Hathway, 1958). In sorghum it has been proposed that tannin polymerization is nonenzymatic (Jacques et al., 1977; Gupta and Haslam, 1979). This nonenzymatic reaction would involve a spontaneous condensation via a carbocation.

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This investigation is concerned with the preharvest changes in tannins in the testa of bird-resistant sorghum grain as well as two enzymes which could be associated with polyphenol polymerization: polyphenol oxidase (PPO) (EC 1.14.18.1) and peroxidase (EC 1.11.1.7). This study was carried out to see what interrelation existed between enzyme activity and tannin deposition.

PPO has been well reviewed (Mayer and Harel, 1979). Sorghum seedlings yielded three fractions of PPO activity when extracts were chromatographed on Sephadex G-100 (Stafford and Dresler, 1972), and a minimum of five bands of peroxidase activity were obtained by electrophoretic separation (Stafford and Bravinder-Bree, 1972). In another study, sorghum seedling peroxidase carried out the aliphatic hydroxylation of (*p*-hydroxyphenyl)acetonitrile which is an intermediate in dhurrin biosynthesis (Liljegren, 1978). From sorghum grain, peroxidase has been isolated but no polyphenol oxidase activity has been reported (Sae et al., 1971).

EXPERIMENTAL SECTION

Bird-resistant sorghum [*Sorghum bicolor* (L.) Moench], cultivar SSK 52, was used to study the preharvest changes while bird-resistant cultivar NK 300 was used to study the properties of the enzymes. Both cultivars were grown at the Plant and Seed Control Division, Horticultural Research Centre, Roodeplaat, South Africa. Samples of SSK 52 were harvested at various stages of development from preflowering (5 days before anthesis) until time of harvest (89 days after anthesis). Ten whole panicles were harvested for each stage of development (as listed in Figure 1), and after hand threshing, the grains were freeze-dried.

After being dried, the grain was mixed and subsamples were ground for 1 min in a Janke and Kunkel mill (a water-cooled coffee mill). The ground grain was extracted at room temperature with 70% aqueous acetone by percolation on a Buchner funnel using 500 mL of 70% ace-

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tone/100 g of ground grain. This extract contained the full complement of extractable polyphenols and all subsequent polyphenol work was done on this extract. The presence of catechin and the dimer was determined by two-dimensional thin-layer chromatography (2-D TLC) in 2-methyl-1-propanol–acetic acid (HOAc)–H_2O (14:1:5 v/v) and 6% HOAc.

Total polyphenols were quantitatively analyzed according to the method of Jerumanis (1972) as modified by Daiber (1975). This method relies on the principle of polyphenols reacting with Fe^{3+} under alkaline conditions. Tannic acid (Merck) was used as a standard. Aliquots of the extract were subjected to fractionation on a Sepharose CL-6B column according to the methods of Kaluza et al. (1980). This procedure separates sorghum polyphenols into three fractions; fraction I contains simple polyphenols while fraction II contains condensed tannins of low molecular weight and fraction III contains high molecular weight tannins.

The enzyme activities of ground grain were determined in the following manner. Insoluble poly(vinylpyrrolidone) (PVP) (0.2 g) was stirred for 10 min in 5 mL of 0.1 M phosphate buffer (pH 5.6 for peroxidase and pH 5.8 for PPO). Ground grain (0.2 g) was added and stirring continued for 30 min. The preparation was then squeezed through nylon muslin and centrifuged at 27000*g* for 20 min. An aliquot of the supernatant was diluted to the desired enzyme activity with buffer of the correct pH. The activity was assayed spectrophotometrically by measuring the disappearance of the chosen substrate at its wavelength of maximum absorption. The compounds used and their wavelength of maximum absorption in nanometers were pyrocatechol (274), catechin (277), and chlorogenic (320), ferulic (304), 2,5-dihydroxybenzoic (320), *p*-hydroxybenzoic (244), gallic (256), and *o*-coumaric acids (268). The substrates and inhibitors were chosen to show enzyme specificity and to distinguish between *o*-diphenol and *p*-diphenol oxidase activities. Cuvettes of 1-cm path length were used and determinations were carried out at 30 °C. To each cuvette 1 mL of enzyme preparation was added and to the sample cuvette 0.2 mL of 1 mM phenolic substrate was added (plus 0.05 mL of 3% H_2O_2 for peroxidase determination) and the volume in both cuvettes was adjusted to 3 mL with buffer.

RESULTS AND DISCUSSION

The amounts of the different types of polyphenols as a function of grain maturity are shown in Figure 1. Fraction I is the fraction from the Sepharose CL-6B column which contains low molecular weight polyphenols which do not precipitate protein. They were the only polyphenols detected during the flowering stage, and they reached a maximum as the grain started to develop at the milk stage. After the milk stage the level of these compounds declined to approximately half their maximum and remained as such up to the time of harvest.

Fractions II and III were the fractions which precipitated protein and appeared to be composed almost exclusively of condensed tannins; because of this they were combined. They were not present in the flowering stage but there was a rapid increase as the grain developed. Observations of the column elution patterns revealed that the tannins not only increased in amounts but their molecular weights also increased as the grain progressed through the early reproductive stages.

TLC studies of sorghum grain extracts failed to detect catechin during the flowering stage. As the grain developed, catechin and the dimer associated with it were present throughout the period of tannin accumulation. As

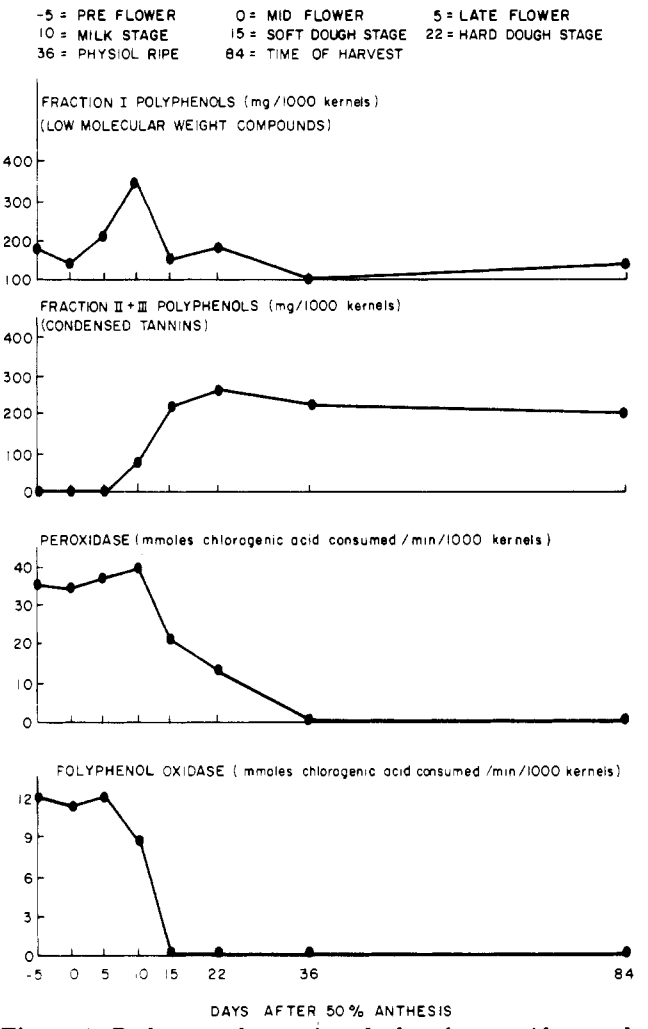

Figure 1. Preharvest changes in polyphenols, peroxidase, and polyphenol oxidase in developing sorghum grain.

soon as the tannin concentration reached a maximum, the catechin and the dimer decreased to only trace amounts and remained as such up to the time of harvest. Since catechin is probably required for tannin synthesis, a decrease in concentration suggested that the main period of tannin synthesis was concluded.

PPO activity which was present during the flowering stage rapidly declined as the grain developed. Peroxidase activity increased slightly during the flowering stage, reaching a maximum in the very immature grain (milk stage). The decline of peroxidase was not nearly as rapid as that of PPO, and peroxidase activity was present until the grain was fully developed (physiologically ripe).

Because of the high tannin content of the grains used in this study, PVP was routinely used in enzyme preparations. Enzyme extractions conducted with and without PVP showed a 3.5-fold increase in activity using PVP. Both phenol oxidizing enzymes isolated from sorghum grain possessed maximum activities at pH values similar to that of PPO isolated from other sources (Mayer and Harel, 1979). Peroxidase had a maximum activity at pH 5.6 and PPO at pH 5.8. The activity of both enzymes dropped to almost zero at pH 4.0 and below.

The oxidative activities of the enzymes on different substrates are presented in Table I. Peroxidase showed a high degree of substrate specificity while PPO possessed both catechol oxidase and cresolase activity. Table II reports enzyme response to various inhibitors; different amounts of the competitive inhibitors were added as shown in Table II. Peroxidase is an iron-containing enzyme and

Table I. Substrate Specificity of Peroxidase and Polyphenol Oxidase from Sorghum Grain

substrate	act., nmol of chlorogenic acid consumed min^{-1} $(g \text{ of ground grain})^{-1}$ peroxidase	polyphenol oxidase
chlorogenic acid	15	15
pyrocatechol	0	10.1
(±)-catechin	0	10.1
gallic acid	0	7.5
o-coumaric acid	0	6.5
ferulic acid	15	1.5
p-hydroxybenzoic acid	0	0.5
2,5-dihydroxybenzoic acid	0	0
tyrosine	0	0

Table II. Effect of Inhibitors on the Oxidation of Chlorogenic Acid by Peroxidase and Polyphenol Oxidase from Sorghum Grain

inhibitor	% inhibition peroxidase	polyphenol oxidase
0.5×10^{-7} M		
rubeanic acid (dithiooxamide)	0	100
sodium diethyldithiocarbamate	89	100
L-(+)-cystine hydrochloride	78	100
EDTA	13	100
NaCN	0	100
phenylhydrazine	100	100
1,3-naphthalenediol	67	100
1×10^{-7} M		
2-nitrophenol	0	38
phenol	0	44
4-nitrophenol	0	44
0.7×10^{-7} M		
2-nitrophenol	0	38
phenol	0	42
4-nitrophenol	0	44
0.3×10^{-7} M		
2-nitrophenol	0	20
phenol	0	22
4-nitrophenol	0	33

as such is only partially inhibited by reagents which selectively complexed with copper. PPO, which possesses copper, suffered complete inhibition when exposed to these agents and phenylhydrazine or 1,3-naphthalenediol.

Since these two compounds inhibit catechol oxidase but not laccase, the PPO from sorghum is a catechol oxidase (*o*-diphenol:O_2-oxidoreductase) and not a laccase (*p*-diphenol:O_2-oxidoreductase) (Mayer et al., 1964; Lerner et al., 1971; Walker and McCallion, 1980).

This pattern of enzyme activity does not appear to correlate well with tannin deposition. Figure 1 shows that the extractable tannin content apparently decreased after it reached its maximum. This confirms the results of Davis and Hoseney (1979) and Price et al. (1979), who found a similar pattern of decrease in tannin. Price et al. found a decrease in the tannin content of nine different sorghum hybrids but the decrease varied from one hybrid to the other.

Gupta and Haslam (1979) believed that there is a short burst of tannin synthesis after which the levels of the products remain approximately constant. This was probably true for the condensed tannins found in sorghum. Goldstein and Swain (1963) suggested that polymerization can continue until the polymers become difficult to extract. This could be the case in sorghum grain and may account for the apparent reduction in tannins as the grain matures.

During this study, sections of the grain at various stages of maturity were prepared for light microscopy. These sections were examined for development of a colored testa which is probably the location of the tannins (Rooney et al., 1979). In the flowering stage no colored testa could be seen, but at the milk stage a very thin colored layer was found. The soft dough stage had a well-defined colored layer which continued to thicken as the grain matured. The extractable tannin content decreased from the hard dough stage until the time of harvest but a similar trend could not be found on the light micrographs. No visible decrease in the area of the colored testa layer was detected. Since this colored layer in the testa did not have any apparent cellular structure (the cells presumably were crushed by the expanding endosperm), the tannins would have the opportunity to complex with other cell components into an insoluble matrix.

As the tannins increased in amount, the activity of the two enzymes decreased (Figure 1). Since all enzyme preparations were carried out in the presence of PVP, this decrease was probably a natural phenomenon and not the result of inactivation by tannins during the extraction process. If the tannins were intimately associated with the enzymes and causing their inactivation, then peroxidase activity would probably reach zero at the hard dough stage when the extractable tannins were at their maximum.

The hypothesis that tannin production is enzyme mediated and involves an oxidation step where a flavan molecule is oxidized by a polyphenol oxidase was reviewed by Brown (1964). Following reaction with guaiacol, both horseradish peroxidase and laccase from *Neurospora crassa* gave high molecular weight compounds which were bonded through carbon to carbon and carbon to oxygen bonds (Gierer and Opara, 1973). Also, it has been suggested that in *Pisum elatius* seed coat, a tannin reaction initiated by catechol oxidase makes the seed coat impermeable (Marbach and Mayer, 1975, 1978).

In sorghum grain, the extractable polyphenol oxidizing enzymes disappeared as the tannins were deposited, instead of remaining at high activities as would be expected if they were involved in tannin biosynthesis. Some peroxidase activity persisted after the initiation of tannin deposition, but Table I shows that catechin was a poor substrate for the enzyme. These patterns of enzyme activity and tannin deposition suggest that the polymerization step in tannin formation was not enzyme mediated in developing sorghum grain.

There appeared to be a definite period of tannin synthesis which did not rely on polyphenol oxidizing enzymes for its final step. These enzymes have been associated with defense mechanisms and help guard against phytopathogens. In sorghum grain, it is possible that once the tannins were present to protect the grain (Swain, 1979), the polyphenol oxidizing enzymes were no longer required for this task and were inactivated.

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Quantitative Determination of Peroxidase in Sweet Corn by Chemiluminescence

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A chemiluminescent method is described for the quantitative determination of peroxidase activity in corn. This method is based on measuring the the light emitted in the oxidation of purporogallin by the peroxidase in the presence of hydrogen peroxide. The photons of the emitted light are counted by a scintillation counter between the 19th and 25th s of the reaction. A linear relationship was established between photon counts and enzymatic activity. The standard error of the means was found to be in the range of 1.0–2.5% of the reading. The method is free from any turbidity intereference, does not require cleanup, and is capable of measuring directly the bound and the soluble fractions of the enzyme.

Peroxidase activity is commonly used as an index for the extent of enzyme deactivation in blanching of vegetables. The development of off-flavor during storage of frozen vegetables has been repeatedly correlated to peroxidase activity (Svensson, 1977; Baardeth, 1978). A fast quantitative method for the determination of this activity is therefore of high practical importance.

Currently, peroxidase activity is determined colorimetrically by enzymatically oxidzed H donors such as *o*-dianisidine ("Worthington Engzyme Manual", 1972), guaiacol (Wise and Morrison, 1971), pyrogallol (Marshall and Chism, 1979), and *o*-phenylenediamine (Vetter et al., 1958). The relatively low sensitivity of these methods requires a proportional increase in sample size for adequate color development. This in turn increases the amount of leached turbid materials which obviously interferes with a direct colorimetric reading. A cleanup procedure is required to overcome this type of interference, especially pronounced in starchy vegetables such as corn. In sweet corn kernels, e.g., an alcoholic precipitation and centrifugation are employed (Vetter et al., 1958) to clarify the sample. There are evidences suggesting the existence of a soluble and a cell wall bound peroxidase fraction (Gordon and Alldridge, 1971; Haard, 1973; Henry, 1975; Yung and Northcoate, 1975). The existence of a bound enzyme fraction casts doubts on the accuracy of peroxidase determination in any system where suspended material is removed.

A possible approach to the determination of peroxidase activity in a blended (and turbid) whole sample may be presented by a chemiluminescence technique. The chemiluminescence determination of peroxidase-mediated oxidation of substrates such as pyrogallol (Nilsson, 1964) and luminol (Maehly, 1955) has been studied. Recently, Halmann et al. (1979) have investigated the chemiluminescence peroxidation of phenolic derivatives such as pyrogallol, resorcinol, phloroglucinol, and purpurogallin. The oxidation mechanism of the latter by H_2O_2 has been studied by Collier (1966).

The purpose of this work was to develop a chemiluminescent technique for the fast and sensitive determination of peroxidase activity directly on the whole blended sample of sweet corn.

EXPERIMENTAL SECTION

Recommended Procedure. Approximately 20 g of corn ear slices (or any other corn tissue) is blended with 10 parts of distilled water for 3 min in a Waring blender. One milliliter of the blended sample is introduced into a scintillation vial containing 2 mL of freshly prepared (every 4 h) purpurogallin solution. The purpurogallin solution is prepared by dissolving 0.02% purpurogallin (Sigma Chemical Co.) in cold 1:10 methanol to 0.18 M, pH 6.5 potassium phosphate buffer. The purpurogallin solution is kept in ice water.

The reaction is started by injecting 1 mL of H_2O_2 (0.12 M in the same phosphate buffer) into the scintillation vial already containing the sample and the purpurogallin so-

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