Contribution of Enzymic Browning to Color in Sugarcane Juice

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The contribution of polyphenol oxidase (PPO) and peroxidase (POD) to enzymic browning in sugarcane juice was investigated. Inactivation of these enzymes with heat resulted in juice of lower color (absorbance measured at 420 nm), but POD was found to be more heat stable than PPO. Salicylhydroxamic acid (SHAM) completely inhibited PPO activity and markedly reduced juice color but had no effect on POD activity. Removal of oxygen in the presence of the substrate chlorogenic acid also stopped color formation. Upon subsequent addition of oxygen, browning continued, indicating that the process was oxygen dependent. Color development in juice was complete after 20–30 min even though PPO was still active. Addition of chlorogenic acid at this point restarted browning, suggesting that color development was limited by the availability of phenolic substrates. Varietal differences were observed in levels of PPO activity, phenolics, and color. There was a correlation between juice color and phenolic content but not between juice color and PPO activity. The sugarcane PPO enzyme was most active with chlorogenic acid. It was not active with *p*-diphenols and was inhibited by SHAM, suggesting that it is a catechol oxidase-type enzyme (EC 1.10.3.1) and not a laccase (EC 1.10.3.2). It is atypical in that it was inhibited by SDS. The results suggest that enzymic browning contributes significantly to color formation in sugarcane juice and that PPO is the major enzyme involved.

INTRODUCTION

Brown pigments are formed during the processing of sugarcane from the initial juice extraction through to crystallization of the raw sugar. The presence of these colored impurities is of considerable significance to the sugar industry as their presence impedes crystallization and results in lower sugar yields, poorer quality sugars, and increased costs of refining (Jimenez and Samaniego, 1981).

At least four different mechanisms are believed to contribute to color formation during raw sugar production (Kort, 1979): (1) melanoidins formed from sugar–amino acid reactions via the Maillard reaction; (2) thermal degradation and condensation reactions of sugars (caramelization); (3) alkaline degradation and condensation reactions of reducing sugars; and (4) oxidative reactions of phenolic compounds. The first three are nonenzymic reactions, whereas the oxidation of phenolic compounds to the chemically more reactive quinones is enzymic and occurs early in the extraction process, when the cane is first crushed. Previous studies suggest that enzymic browning may contribute significantly to color in cane juice. Smith (1976) found that heating cane to 80–90 °C prior to crushing resulted in a 47% reduction in average juice color, and Tu (1977) observed a similar reduction in both cane juice and raw sugar color when the juice was made alkaline to inhibit enzyme activity.

Both polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (POD, EC 1.11.1.7) have been implicated in enzymic browning of plant tissues (Vamos-Vigyazo, 1981). Polyphenol oxidase is widely distributed in the plant kingdom (Mayer and Harel, 1979). It is a copper-containing enzyme which catalyses the ortho-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Mayer and Harel, 1979). The highly reactive quinones thus formed can polymerize to form the red, black, and brown pigments associated with the browning of plant tissues. Normally, PPO is separated from its phenolic substrates, which are located in the vacuole, so that browning only occurs when cells are damaged and compartmentation is lost. As yet the physiological function of PPO has not been established, although it has been associated with disease resistance (Vaughn *et al.*, 1988).

Peroxidase is an iron-containing enzyme capable of oxidizing phenolics to quinones in the presence of hydrogen peroxide. Like PPO, the physiological role of peroxidase in plants is not well understood but it has been implicated in a number of primary and secondary metabolic functions including lignin biosynthesis, ripening and senescence, ethylene biosynthesis, hormone balance, and membrane integrity (Vamos-Vigyazo, 1981). Lagrimini (1991) produced transformed tobacco plants overexpressing peroxidase which showed rapid browning in response to wounding, but the role of peroxidase in normal enzymic browning is still not well established.

An active PPO with a high specificity for chlorogenic acid has been isolated from sugarcane leaf tissue (Coombs *et al.*, 1974). Subsequent inhibition and heat inactivation studies suggested PPO contributed significantly to color formation in sugarcane (Gross and Coombs, 1976b; Coombs and Baldry, 1978; Goodacre *et al.*, 1980). The presence of POD in sugarcane and its properties have been studied (Alexander, 1966); however, its contribution to color formation has not been investigated.

The aim of this study was to determine the contribution of enzymic browning to color formation in sugarcane juice and to determine the relative contributions of each of these enzymes to browning.

MATERIALS AND METHODS

Plant Material. Sugarcane varieties Q87 and Q96 were propagated from clonal setts and grown in a heated glasshouse with an average day temperature of 30 °C and a night temperature of 17 °C. The canes 81C236, Q87, H56-752, 81C337, 81C497, Q96, 81C542, 81C558, and 81C509 for the varietal studies (Figures 2 and 3) were grown at the Bureau of Sugar Experiment Station (BSES), Mackay Queensland, under field conditions and harvested Sept 5, 1991.

Tissue Extraction and Enzyme Assays. All experiments were repeated several times with cane tissue showing differing degrees of browning; unless otherwise stated the data shown are representative of a typical situation. Individual measurements were replicated two to three times. Lengths of cane were

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Table 1. Effect of Temperature on PPO and POD Activities, Color, and Total Phenolics in Sugarcane Juice[a]

buffer temp (°C)	final temp (°C)	absorbance 420 nm	PPO activity (μmol of O_2 min^{-1} gFW^{-1})	POD activity ($\Delta A470$ min^{-1} gFW^{-1})	phenolics (mg gFW^{-1})
25	25	0.792	2.1	76	1.16
50	41	0.830	2.0	97	1.19
65	48	0.715	1.1	104	1.24
80	58	0.467	0.2	63	1.36
100	72	0.230	0.0	47	1.75

[a] Cane of the variety 81C337 was homogenized in buffer at the temperatures indicated, and the final temperature of the extract was determined after homogenization.

sliced transversely into 2–3-m-thick disks using a Berkel food processor and then ground with polytron blender in 4 volumes of ice-cold 100 mM NaH_2PO_4 (pH 5.0) containing 1 mM $MgCl_2$.

For the inhibition studies, the specific inhibitors were added to the extraction buffer and the tissue homogenized in the presence of the inhibitors. Where cane tissue was heat treated, sliced cane was added directly to buffer of the appropriate temperature and immediately homogenized. The homogenate was filtered through Miracloth and used for enzyme assays. Polyphenol oxidase activity was measured as oxygen uptake in a Hansatech oxygen electrode at 25 °C. The reaction chamber contained 50 mM NaH_2PO_4 (pH 6.0) and extract in a total volume of 1 mL, and the reaction was initiated by adding chlorogenic acid to a final concentration of 2 mM (unless stated otherwise). A pH of 6.0 was found to be optimal for the reaction (data not shown). Peroxidase activity was monitored spectrophotometrically at 470 nm and 25 °C. The reaction cell contained 0.56% (v/v) guaiacol, 50 mM NaH_2PO_4 (pH 6.0), and extract in a total volume of 1 mL. Hydrogen peroxide was added to a final concentration of 0.3% (v/v) to initiate the reaction. No change in color was detected in the absence of added peroxide.

Juice Color Determination. Unless stated otherwise, cane extracts were allowed to stand for at least 40–60 min, by which time browning was complete, and then centrifuged at 40000*g* at 4 °C for 10 min. The supernatant was filtered through a 0.45-μM Millipore filter and color assayed spectrophotometrically at 420 and 720 nm. The absorbance at 720 nm reflects the degree of turbidity and was subtracted from the absorbance measured at 420 nm. Where the change in color of juice extracts was measured over time (Figures 1 and 4), samples were taken and SHAM was added to a final concentration of 2.5 mM to prevent further oxidation due to PPO. This inhibitor stop method was found to prevent any further color development in the juice.

Phenolics. Phenolics were measured in aqueous extracts (Table 1), made as described for the measurement of color. For the varietal studies (Figure 3) total phenolics were measured in methanolic extracts. Sliced cane was ground with a Polytron blender in 4 volumes of 80% methanol and then centrifuged at 40000*g* for 10 min. The supernatant was collected and the pellet reextracted in methanol and centrifuged again. The supernatants were combined and filtered through a 0.45-μm Millipore filter. Total phenolics were measured following the method of Rathjen and Robinson (1992), using the Folin–Ciocalteu reagent. Phenolic levels are expressed in gallic acid equivalents per gram fresh weight. All measurements were made in duplicate.

RESULTS AND DISCUSSION

To investigate the contribution of polyphenol oxidase and peroxidase to the browning of sugarcane juice, cane tissue was extracted at various temperatures, and juice color, total phenolics, and remaining activity of the two enzymes were determined (Table 1). There was some heat loss upon addition of cane to the extraction buffer and also during homogenization so both the initial temperature of the buffer and the final temperature of the extract are given in Table 1.

The color of the sugarcane juice decreased with increasing extraction temperature. For cane extracted in buffer at 100 °C, color was reduced by 71% compared to the control cane extracted at 25 °C. There was also an increase in the level of phenolics remaining in the juice following extraction at higher temperatures, which is consistent with phenolics acting as substrates for the browning reaction in cane juice. Polyphenol oxidase activity decreased with increasing temperature, and no PPO activity was detected in juice extracted at 100 °C. Peroxidase was less sensitive to the heat treatment, and almost half of the activity still remained in juice extracted at 100 °C. This is consistent with previous reports on peroxidase, which indicate that it is a relatively heat-stable enzyme and is thus widely used as an index of blanching and other heat treatments (Burnette, 1977).

Table 2. Effect of Inhibitors on PPO and POD Activities and Color in Sugarcane Extracts[a]

inhibitor	PPO activity (%)	POD activity (%)	absorbance at 420 nm (%)
control	100	100	100
SHAM	0	103	30
heat	0	55	35
tropolone	23	79	36
DIECA	7	62	51

[a] All values are expressed relative to the control where PPO activity was 3.0 μmol of O_2 min^{-1} gFW^{-1}, POD activity 98 $\Delta A470$ min^{-1} gFW^{-1}, and the A420 was 0.6 absorbance units. Salicylhydroxamic acid (SHAM) and tropolone were present at a final concentration of 1.7 mM, DIECA was 8 mM. For the heat treatment cane was extracted at 100 °C. The variety was 81C337.

The inhibition of color formation in cane juice by heat suggests that enzymic browning does contribute significantly to color. The progressive decrease in color measured at 420 nm with increasing extraction temperature indicates that more than 70% of the color formed initially in cane extracts could be attributed to enzymic browning reactions. This is in agreement with previous studies where heat treatments inhibited color formation in cane juice (Goodacre *et al.*, 1980; Smith, 1976). Furthermore, the data in Table 1 suggest that PPO in sugarcane juice is more sensitive than peroxidase to inhibition by heat treatment.

The relative contribution of PPO and POD to color formation was further characterized using enzyme inhibitors. Salicylhydroxamic acid (SHAM) is a powerful noncompetitive inhibitor of polyphenol oxidase (Allan and Walker, 1988). Diethyl dithiocarbamate (DIECA) is also an efficient inhibitor of PPO and acts by chelating copper in the active site of the enzyme (Anderson, 1968). Tropolone is a slow binding competitive inhibitor, structurally analogous to the *o*-diphenolic substrates of PPO, as well as an effective copper chelator (Valero *et al.*, 1991). In the presence of hydrogen peroxide, tropolone acts as a substrate for peroxidase rather than an inhibitor and can be used to distinguish between the reactions of polyphenol oxidase and peroxidase (Kahn, 1985).

These inhibitors were added to the grinding medium prior to extraction of the sugarcane, and then juice color and the activities of PPO and POD were determined (Table 2). A heat treatment where cane was extracted in grinding medium heated to 100 °C was also included for comparison. All of the treatments inhibited PPO and decreased juice color. In contrast, POD activity was not inhibited in all cases and the extent of inhibition was always less than for

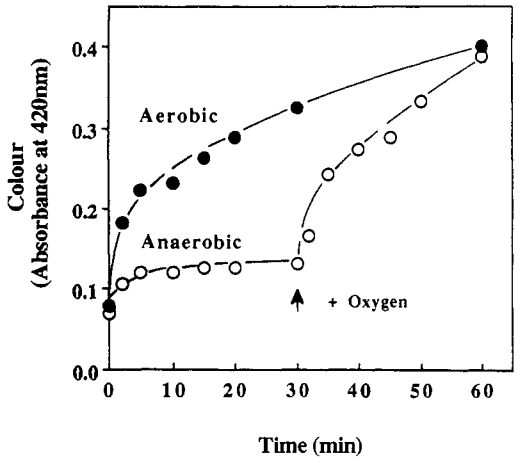

Figure 1. Color development in cane juice in the presence and absence of oxygen. Oxygen was added back to the anaerobic sample after 30 min.

PPO. PPO activity was completely inhibited by SHAM and there was a marked decrease in juice color yet there was no effect on POD activity (Table 2). Juice color in the sample with tropolone was lower than might be expected from the measured PPO activity, which was 23% of the control. This may indicate that tropolone inhibition of PPO in the juice, where the level of phenolic substrates may be limiting, was greater than that determined in the PPO assay with saturating levels of substrate.

Enzymic browning catalyzed by polyphenol oxidase is an oxygen-dependent reaction, unlike the oxidation catalyzed by peroxidase. If PPO is a major contributor to browning, removal of oxygen from the system should inhibit color formation. Cane (variety Q96) was gound in the cold to minimize PPO activity, and the extract was then degassed to remove oxygen. Nitrogen was blown over the extract, and the oxygen concentration was monitored with an oxygen electrode. When the oxygen tension was near zero, substrate (chlorogenic acid) was added and half the extract was exposed to air while the other half was kept under nitrogen.

There was an initial increase in color in the anaerobic sample which may have resulted from residual oxygen in the sample, but there was no further increase in color after the first 5 min, whereas color of the extract exposed to air increased (Figure 1). After 30 min the extract under nitrogen was exposed to air, resulting in a sharp increase in A420, and after 1 h the two extracts had achieved the same A420 (Figure 1). Thus, color development was severely inhibited in the absence of oxygen and was restarted upon addition of oxygen suggesting that under normal extraction conditions enzymic browning is predominantly an oxygen-dependent reaction in sugarcane juice.

There are varietal differences in the susceptibility of sugarcane juice to brown. A survey of commercial sugarcane varieties was undertaken to determine the variation in juice color and PPO activity. Nine varieties grown at Mackay Queensland were assayed for polyphenol oxidase activity, total phenolics, and absorbance at 420 nm. There was wide variation in all three parameters, and the results presented in Figures 2 and 3 are for three separate samples of cane from each variety. A least-squares linear regression showed only a weak correlation ($R^2 = 0.17$; p value = 0.037) between color and PPO activity (Figure 2). There was, however, a strong correlation ($R^2 = 0.76$; p value < 0.0001) between total phenolics extracted and A420 measured at pH 5.0 (Figure 3).

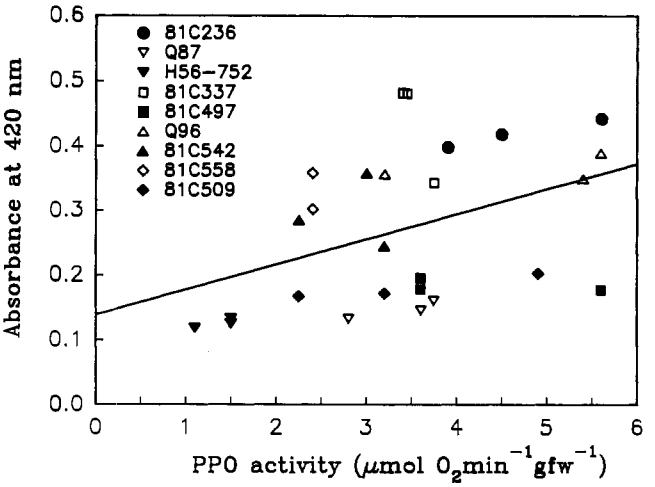

Figure 2. Relationship between PPO activity and color in nine commercial varieties. Each point represents the PPO activity and color determined in an extract from a single cane. Extracts from three individual canes were made for each variety. The line indicated had a correlation coefficient (R^2) of 0.17.

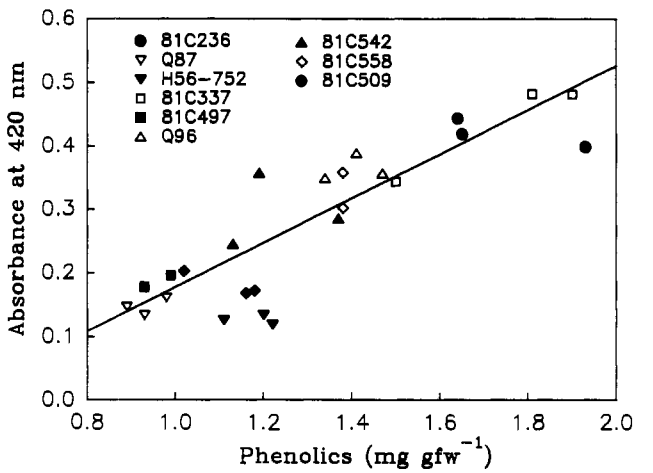

Figure 3. Relationship between phenolics and color in nine commercial varieties. Each point represents the phenolics and color determined in an extract from a single cane. Extracts from three individual canes were made for each variety. The line indicated had a correlation coefficient (R^2) of 0.76.

Varieties may have a lowered potential for enzymic browning as a result of decreased levels of the phenolic substrates, lower levels of the enzymes involved, or the presence of endogenous inhibitors. The nine cane varieties used in the varietal studies (Figures 2 and 3) are commercial hybrids and have been bred to have desirable traits such as high yield, high sucrose, and disease resistance. The poor correlation between PPO activity and color (Figure 2) may well be due to tissue heterogeniety and the difficulty in sampling uniform cane material. The strong correlation between color and total phenolics (Figure 3), however, suggests that this is not the case and that availability of phenolic substrates limits the browning reaction in sugarcane juice. In apples, Harel *et al.* (1966) also found a good correlation between browning and *o*-diphenol content, but the correlation with catechol oxidase activity was poor.

The possibility that levels of phenolic substrates determine the extent of color formation in sugarcane juice was investigated further. Cane tissue of Q96 extracted at 4 °C was warmed to 25 °C, and color development (A420) was monitored over time. Color development was rapid initially but reached a plateau after 30 min (Figure 4), even though 75% of the initial PPO activity remained.

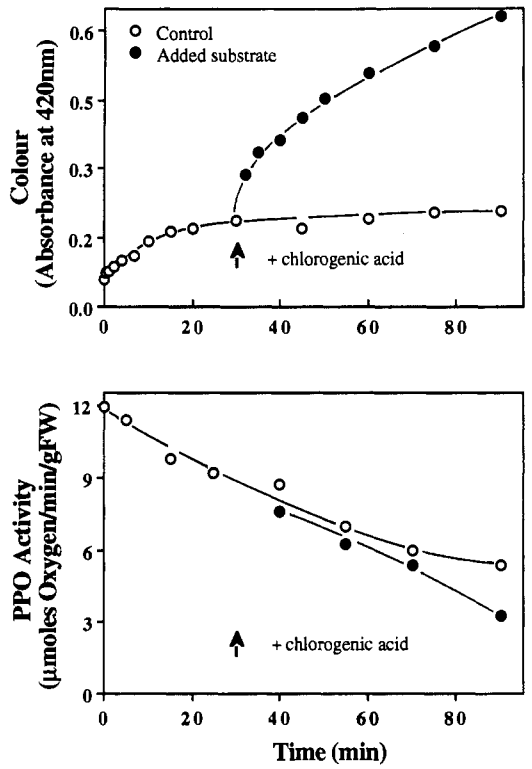

Figure 4. Color development in cane juice (variety Q96) over time. Chlorogenic acid was added to half the extract to a final concentration of 1 mM after 30 min, as indicated by the arrow. Samples were taken over time and immediately assayed for PPO activity and color as described under Materials and Methods.

Addition of 1 mM chlorogenic acid at this point resulted in a rapid increase in A420, suggesting that a lack of appropriate substrate was limiting the color reaction. After 90 min, color development of the extract with added chlorogenic acid had slowed considerably. PPO activity declined substantially over time, suggesting it was being inhibited by its product or some other compounds in the extract. Sapis *et al.* (1983) reported a similar finding for grapes, showing the potential for browning to be substrate limited. Substrate concentration also governed the degree of actual browning of different apple varieties (Walker, 1964).

Further studies were undertaken to characterize sugarcane polyphenol oxidase. The name polyphenol oxidase is commonly used to describe the catechol oxidase type enzymes (EC 1.10.3.1) but also includes the laccase enzymes (EC 1.10.3.2). Substrate specificity is diagnostic of the type of PPO enzyme involved. Laccases can oxidize both *p*- and *o*-diphenols while catechol oxidases oxidize only the *o*-diphenols (Mayer and Harel, 1979). Sugarcane PPO readily oxidized chlorogenic acid, and to a lesser extent some other *o*-diphenols such as 4-methylcatechol, catechol, and caffeic acid (Table 3). The enzyme had low activity with monophenols (ferulic acid, tyrosine, and *p*-cresol), and oxidation of the *p*-diphenol, toluquinol, and *p*-phenylenediamine was less than 3% of the activity with chlorogenic acid as shown in Table 3. Cane juice extracts desalted on Sephadex G-25 showed the same substrate specificity as the crude preparation (data not shown), suggesting that there was not significant interaction with the endogenous phenolics.

In healthy plant cells, PPO is localized in the chloroplast where it is bound to the thylakoid membrane (Vaughn *et al.*, 1988). The sugarcane PPO is unusual in that is appeared to be readily soluble in both leaf and cane extracts (data not shown). In many plants, PPO exists in a latent

Table 3. Substrate Specificity of Sugarcane PPO[a]

substrate	rel PPO activity	substrate	rel PPO activity
chlorogenic acid	100	ferulic acid	4
catechol	9	tyrosine	0
caffeic acid	19	*p*-cresol	1
4-methylcatechol	22	*p*-phenylene-diamine	2
catechin	5		
dihydroxyphenyl-alanine	0	toluquinone	1

[a] All substrates were present at a final concentration of 2 mM, except for dihydroxyphenylalanine, which was 1 mM. Activity was measured in the variety Q87 and expressed relative to that with chlorogenic acid.

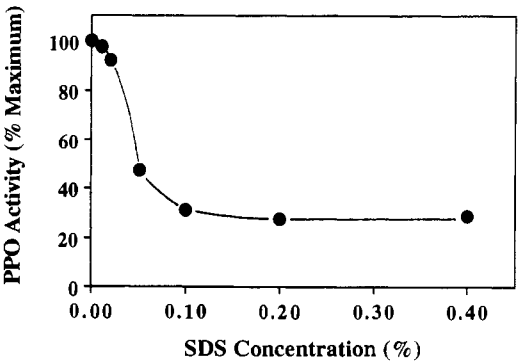

Figure 5. Effect of SDS on PPO activity in cane juice. Extracts were assayed at 25 °C in the presence of SDS.

state and can only be detected following activation by anionic detergents, fatty acids, treatment with proteases, or acid treatment (Mayer, 1987). In plant extracts SDS is commonly used to activate PPO enzymes (Flurkey, 1986). Extracts were assayed in the presence of SDS to ensure that PPO in sugar cane was fully activated. SDS failed to increase measured activity and actually inhibited PPO activity at quite low concentrations (Figure 5). At 0.1% SDS, PPO activity was inhibited by approximately 70%.

The sugarcane PPO utilized a limited range of phenolic compounds with the *o*-diphenol chlorogenic acid, by far the preferred substrate (Table 3). Both chlorogenic acid and its hydrolysis product, caffeic acid, have been identified in sugar cane tissue (Gross and Coombs, 1971). Inhibition of PPO in cane juice by SHAM (Table 2) and its inability to oxidize *p*-diphenols (Table 3) indicate that it is a catechol oxidase-type PPO enzyme and not a laccase. It is, however, atypical of catechol oxidase-type enzymes in that it was inhibited by SDS (Figure 5).

The inhibition of color formation in juice by heat (Table 1), inhibitors of PPO (Table 2), and anaerobic conditions (Figure 1) indicates that enzymic reactions make a major contribution to color formation in sugarcane juice. The oxygen requirement for color formation (Figure 1) and the observation that SHAM, which totally inhibited PPO without affecting POD, reduced color formation to the same extent as boiling (Table 2), are strong evidence for the dominant role of PPO in enzymic browning in cane juice. It appears that, although cane juice exhibits significant peroxidase activity which can cause enzymic browning, this enzyme does not contribute to color formation under normal extraction conditions, possibly because of insufficient levels of hydrogen peroxide.

Under normal extraction conditions it appears that polyphenol oxidase and not peroxidase is responsible for enzymic color formation in cane juice. It is also apparent

that the formation of colorants in sugarcane juice is the result of a complex interaction between the enzyme, the concentration, and type of phenolic substrate, and possibly other reactive compounds present in the juice. The results shown in Figures 2–4 indicate that the availability of phenolic substrates may determine the extent of the browning reaction in cane juice.

The results of this study suggest that inactivating the PPO enzyme would result in a significant decrease in cane juice color. Selection of low browning varieties on the basis of PPO activity does not appear to be feasible due to the weak correlation between these parameters (Figure 2). Chemical inhibition of PPO is possible but may not be an economic or desirable option for a food product. Thermal inactivation of the PPO is also possible as it does not appear to be very heat tolerant, although white sugar produced from steamed cane has been reported to have a strong and persistent taste (Coombs *et al.*, 1980). Further studies are underway to characterize the sugarcane PPO enzyme with the aim of decreasing its activity during processing or producing varieties with low levels of PPO or of its specific phenolic substrates.

ABBREVIATIONS USED

DIECA, diethyl dithiocarbamate; PPO, polyphenol oxidase; POD, peroxidase; SHAM, salicylhydroxamic acid; SDS, sodium dodecyl sulfate.

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