Intervention of Phenolic Compounds in Plum Technology. 2. Mechanisms of Anthocyanin Degradation

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Anthocyanins disappear during the initial hours of drying of d'Ente plum (*Prunus domestica*, var. d'Ente). The use of model solutions indicates that d'Ente plums do not appear to possess enzymes capable of acting directly on anthocyanins. However, glutathione was used to show that anthocyanins can be transformed by a coupled oxidation mechanism involving quinones formed from chlorogenic acid under polyphenoloxidase dependence. HPLC was found to be useful in the simultaneous monitoring of the various constituents of the reaction medium.

During the drying of d'Ente plums, which lasts 24 h on average, the degradation of anthocyanins takes place at a high rate and during the first hour. Rate disappearance increases with temperature (Raynal et al., 1986). It thus appeared interesting to investigate further the phenomena involved in this disappearance. Much work has been carried out on the enzymatic oxidation of anthocyanins. For example, Wagenknecht et al. (1960) attributed browning of cherries to the action of an anthocyanase that only functioned in the presence of O_2 and whose action was reinforced by catechol. In fact, the general term anthocyanase is given to the enzymes responsible for the oxidation of anthocyanins, but this activity can be separated into two groups: a glycosidase activity thought to release aglycon from anthocyanin [this unstable aglycon may become transformed spontaneously into colorless derivatives (Huang, 1955; Peng and Markakis, 1963)]; an activity resulting from the action of polyphenoloxidase (PPO) on anthocyanins in the presence of an *o*-diphenol, as reported in numerous fruits such as cherries (Van Buren et al., 1960; Pifferi and Cultrera, 1974) and grapes (Skalski and Sistrunk, 1973).

Plum exocarp possesses high PPO activity, and the dihydroxycinnamic acids that are preponderant in this part of the fruit are the preferential substrates of the oxidase. The existence of a reaction mechanism involving these phenol acids as intermediates for oxidation of anthocyanins is thus fully possible.

In comparison with spectrophotometric measurements, the originality of the method used (HPLC) lies in the fact that it enabled specific assaying of the fate of several substrates during the same enzymatic reaction.

MATERIALS AND METHODS

Model System of the Degradation of Anthocyanins. The reaction model of the degradation of anthocyanins included solutions of cyanidin 3-rutinoside (Fluka) (Cn3R, the main anthocyanin in plum exocarp) and chlorogenic acid (Fluka) at 0.16 and 0.28 mM, respectively, in McIlvaine buffer at pH 4.25, together with crude enzymic extract of the exocarp at the ratio 1:1:0.5, with the extract prepared according to Moutounet (1976). When glutathione (Sigma) was added to the reaction medium, its concentration in the latter was 0.47 mM. The reaction was sometimes inhibited by 50 mM phenylthiourea, which prevents the functioning of copper enzymes such as polyphenoloxidases. The reaction preparation was incubated at 30 °C.

Analytical Conditions of Constituent Assays. HPLC analysis of 20 μL of the reaction mixture after various incubation times was monitored with a Waters Associates apparatus, consisting of an AGC 680 controller, a Data Model 680 recorder, two pumps (Models 45 and 6000), and a Model 440 double-detection absorption monitor. The separation of the constituents of the medium was carried out on a Bondapak C_{18} W_3 column (7.8 × 300 mm) fitted with a Brownlee C_{18} 30 × 4.6 mm precolumn. The elution gradient was linear to reach 100% of the solvent formic acid–methanol–water (10:50:40, v/v) in the initial solvent formic acid–water (10:90, v/v) in 20 min with a flow rate of 1.5 mL/min. The column temperature was maintained constant at 30 °C by a thermostatically controlled oven. The solvents were degassed ultrasonically and then filtered on Sartorius 0.45-μm regenerated cellulose filter before use. Cn3R and chlorogenic acid were detected simultaneously at 280 and 546 nm and quantified by the Data Module. The results were expressed as a percentage of the initial concentration of each substrate.

RESULTS AND DISCUSSION

Thermal Degradation of Cn3R. The first step taken was to study the effect of temperature on the degradation of Cn3R. A solution of Cn3R at 0.16 mM was incubated in McIlvaine buffer at pH 4.25 at temperatures of 55, 75, and 95 °C. Degradation of anthocyanins increased with the rise in temperature (Figure 1). Indeed, in vivo, the color of the anthocyanins and their stabilization are caused by a combination of local pH and copigmentation phenomena, which in particular involve flavonols or tannins (Van Buren, 1970). A solution of anthocyanins was subjected in vitro to equilibrium reactions dependent on pH and temperature. The disappearance of Cn3R with time was caused by endothermic reactions leading to colorless carbinol and chalcones. However, the effect of heat does not account for the strong degradation of anthocyanins observed in plum epicarp (Raynal et al., 1986). Indeed, after 1 h, only 12.5%, 24.5%, and 40% Cn3R had been degraded at 55, 75, and 95 °C. At 30 °C, under the experimental conditions of the study of the reaction model, this degradation was negligible.

Degradation by Enzymatic Extract of Cn3R and Chlorogenic Acid Examined Separately. Chlorogenic acid underwent considerable degradation in the presence of enzyme extract; only 25.5% of the acid remained after 20 min (Figure 2A). The rate of oxidation fell increasingly with time, although there was still chlorogenic acid in the medium. This was probably caused by the inhibitory reaction of the oxidation products formed by PPO (Sanderson, 1965). It should be noted that chlorogenic acid was not hydrolyzed since no free caffeic acid was detected by HPLC. However, Cn3R did not undergo degradation at the enzymatic rate in the presence of exocarp extract (Figure 2A). Crude enzymatic extract thus had no direct

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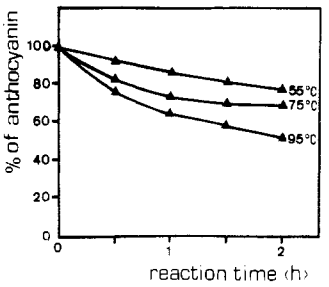

Figure 1. Degradation of a solution of cyanidine 3-rutinoside with time as a function of temperature.

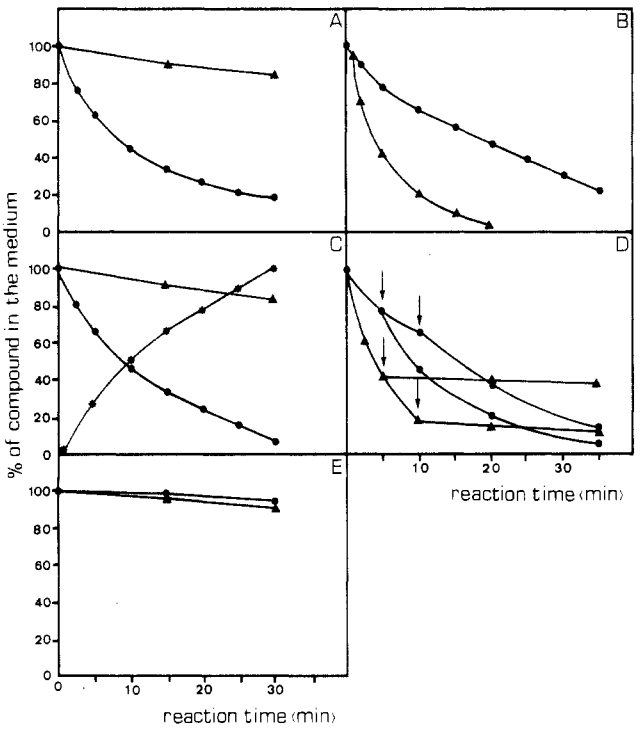

Figure 2. Evolution of chlorogenic acid (●) and cyanidine 3-rutinoside (Cn3R) (▲) in the presence of crude extract of d'Ente plum polyphenoloxidase under the following conditions: (A) Cn3R + enzymatic extract, chlorogenic acid + enzymatic extract; (B) chlorogenic acid + Cn3R + enzymatic extract; (C) chlorogenic acid + Cn3R + enzymatic extract + glutathione, (*) glutathionylchlorogenic derivative; (D) chlorogenic acid + Cn3R + enzymatic extract + glutathione after 5 or 10 min (→); (E) chlorogenic acid + Cn3R + enzymatic extract + enzymatic inhibitor.

effect on the degradation of anthocyanins.

Degradation of Cn3R by a Mixture of Enzymatic Extract and Chlorogenic Acid. In the presence of chlorogenic acid and enzymatic extract, Cn3R was very rapidly degraded and could not be detected by assay after 20 min (Figure 2B). However, degradation of chlorogenic acid was quantitatively less important when it was alone in the presence of enzymatic extract (46.5% remained after 20 min instead of 25%). It would therefore seem that degradation of Cn3R is strongly stimulated in the presence of chlorogenic acid. It should be noted that no degradation occurs when enzymatic extract heated for 5 min at 90 °C is added to a chlorogenic acid–Cn3R mixture (results not described). Degradation of the two compounds is thus clearly of enzymatic origin.

Effect of Glutathione. A component able to bind to anthocyanins was sought in order to confirm the participation of quinones in their oxidation. The sulfhydryl groups are known to inhibit PPO by binding to quinones formed enzymatically and thus preventing them from participating in secondary browning reactions and/or from reacting directly with the enzyme (Kahn, 1985). For example, Roberts (1959) studied the oxidation of catechin in the presence of cysteine or glutathione and showed that when excess cysteine is produced, oxidation of catechin results in colorless substances with the structure of a cysteinylcatechin derivative. Likewise, Graham et al. (1978) showed that dopaquinone reacted with free cysteine or cysteinyl residues of glutathione to form 2-*S*-cysteinyl-DOPA. In grape juice, Cheynier and Van Hulst (1988) showed that the product of oxidation of caftaric acid combined with glutathione to form a 2-*S*-glutathionylcaffeoyltartaric (GRP) derivative. The effect of glutathione was studied first on enzymatic activity and also on the products formed by adding glutathione to the reaction medium in larger quantities than chlorogenic acid (chlorogenic acid:glutathione = 1.5).

Effect on the Degradation of Chlorogenic Acid Alone. The presence of glutathione did not modify the degradation kinetics of chlorogenic acid (Figure 2C). At the concentration used, this substance does not therefore appear to act directly either on the enzyme or on chlorogenic acid. Nevertheless, chlorogenic acid had practically disappeared after 30 min, and the inhibition of PPO by the quinones formed, which was observed in the degradation kinetics of chlorogenic acid (Figure 2A), did not occur. The quinones formed were therefore blocked by glutathione. Indeed, a compound appeared in the medium simultaneously with the disappearance of chlorogenic acid; under HPLC, this compound eluted just after chlorogenic acid. This compound had not been analyzed, but given the composition of the medium and the analogy with similar research (Cheynier and Van Hulst, 1988), this substance is certainly the glutathionylchlorogenic derivative. When Cn3R was added after glutathione, the latter was very little degraded (Figure 2C); degradation of chlorogenic acid remained similar.

Effect on Cn3R–Chlorogenic Acid Mixture. When glutathione was added to the complete reaction mixture after 5 or 10 min of reaction, chlorogenic acid was degraded more rapidly (Figure 2D). In contrast, degradation of Cn3R was blocked immediately, and the Cn3R contents remained steady until 35 min. The quinones formed thus seem to be responsible for the oxidation of anthocyanins; the latter were no longer degraded when quinones were blocked by glutathione. The increase in the rate of degradation of chlorogenic acid may have been caused by the lifting of the inhibition of PPO by quinones, the latter having been blocked by glutathione. In addition, the quinones formed enzymatically could no longer be reduced to chlorogenic acid by the anthocyanin-coupled oxidation mechanism.

Action of an Enzymatic Inhibitor. Phenylthiourea inhibits Cu^{2+} enzymes (Mathew and Parpia, 1971; Kahn, 1977). Chlorogenic acid was not oxidized by the crude enzymatic extract when the medium contained the inhibitor (Figure 2E). In addition, this inhibition prevents degradation of Cn3R. Oxidation of the chlorogenic acid responsible for oxidation of Cn3R is thus certainly enzymatic and catalyzed by polyphenoloxidases.

CONCLUSIONS

The use of model solutions has made it possible to understand the mechanism involved in the disappearance of anthocyanins from plums at the beginning of drying. The main anthocyanin in the exocarp and crude enzymatic extract of plums were chosen for this work. Use of model solutions showed that anthocyanin degradation is dependent on the presence of quinones. This mechanism

demonstrated in vitro is probably enhanced in plums under natural conditions. Indeed, polyphenoloxidases and o-diphenols come into contact very rapidly at the beginning of drying through a cellular decompartmentation phenomenon (Raynal et al., 1985). In addition, polyphenoloxidase is very active at pH close to that of plums and displays great affinity for o-diphenols (Raynal et al., 1986). These compounds are oxidized rapidly and the products of oxidation are able to oxidize anthocyanins rapidly. This degradation is enhanced by their sensitivity to heat.

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Inhibition of Cucumber Tissue Softening in Acid Brines by Multivalent Cations: Inadequacy of the Pectin "Egg Box" Model To Explain Textural Effects[1]

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Low concentrations of added calcium ions were shown to effectively reduce the first-order softening rate of cucumber (*Cucumis sativus*) mesocarp tissue stored in acid brines with 1.5 M NaCl. Softening rates of cucumber tissues without added calcium were hyperbolically correlated with the natural level of calcium in the cucumber (1.8–8.2 mM). The relationship between inhibition of softening rates and the concentration of added calcium was also hyperbolic. Inhibition of softening by added multivalent ions was in the relative order Ca ~ Sr > Ba ≫ Cu > Al. Cd, Co, Zn, and Mg were not effective softening inhibitors. All 12 lanthanide ions tested inhibited tissue softening. On a molar basis, La, Ce, Pr, Nd, Sm, and Eu had a greater inhibitory effect than calcium. Calculated low frequencies of calcium/pectin cross-linkages, the pattern of softening inhibition by other metal ions, and the inability of Cd ions to affect softening inhibition by calcium all suggested that the egg box model does not provide an adequate explanation for the observed textural effects of multivalent metal ions on cucumber tissue.

Recently, it was observed that there is a salt-softening effect when NaCl or other alkali-metal salts are added to acidified cucumber tissue (McFeeters et al., 1989). This effect is similar to that observed in a number of low-acid vegetables (Van Buren, 1986), but the softening mechanism involved may be different due to the low pH (Doesburg, 1965). Since calcium has been found to inhibit softening of cucumber tissue in a variety of conditions (Buescher et al., 1979, 1981; Fleming et al., 1978; Thompson et al., 1979; Tang and McFeeters, 1983; McFeeters et al., 1985), it was of interest to determine the effect of calcium ion under conditions in which salt softening occurred.

Calcium appears to help maintain cellular adhesion in fresh potato tissue (Linehan and Hughes, 1969) and to hold

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