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Glutathione and Cinnamic Acid: Natural Dietary Components Used in Preventing the Process of Browning by Inhibition of Polyphenol Oxidase in Apple Juice

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Consumer demands for 'freshness' in processed foods has been given increasing attention by food processing industries by searching for minimally processed products. Polyphenol Oxidase (PPO) mediated browning is a major cause of undesirable flavors and nutritional losses in fruit juices. Here the anti-browning efficiency of glutathione (GSH, reduced form) and cinnamic acid (CA) in apple juice is evaluated. It was observed that the rate of the browning reaction could be efficiently delayed using GSH and CA, which act as inhibitors of PPO. Kinetic studies confirm that GSH and CA are non-competitive and competitive inhibitors of PPO respectively.

Keywords: Polyphenol Oxidase; Glutathione; Cinnamic acid; Browning; Apple juice

INTRODUCTION

The enzymatic browning reaction occurs in many fruits and vegetables when certain phenolic compounds, in cut, bruised or wounded tissues, undergo oxidation to *o*-quinone, a reaction catalyzed by the enzyme PPO (EC 1.14.18.1).[1] Quinones then polymerize either with themselves, or with amino acids or proteins to form black or red polymers called melanins (Figure 1),[2] which negatively influence the quality and marketability of commercial products.[3]

The usual approach to inhibit both enzymatic and nonenzymatic oxidative browning in foods has been to employ sulfites.[4] Alternative means of controlling enzymatic browning in raw fruits and vegetables are required by the food industry due to restrictions in the use of sulfites, because of health concerns.[5] Amongst the chemical approach to inhibit enzymatic browning, probably ascorbic acid (Vitamin C) is the most frequently studied alternative to sulfites. This vitamin is a highly effective inhibitor of enzymatic browning, primarily because of its ability to reduce quinones back to phenolic compounds before they can undergo further reaction to form pigments.[6] Another frequently used compound is citric acid which acts as a chelating agent and acid generator, both functionalities inhibiting PPO by chelating the Cu cofactor as well as decreasing the overall pH below the optimum for PPO.[7] Substituted resorcinols, especially 4-hexyl resorcinol has been reported as a potent inhibitor of apple browning.[8] Recently a combination (1:1) of β-cyclodextrin (a complexing agent) and L-ascorbate -2-triphosphate has proved to be effective in retardation of browning in apple juice.[9] Thiol-containing compounds, such as cysteine are reducing agents that inhibit PPO mediated browning in fruit juices.[10] Glutathione (GSH, reduced form), a potent cellular reductant, has been reported as an effective inhibitor of browning in fruit juices.[10,20] Phenolic acids, including cinnamic acid (CA) and its derivatives, which are known to possess antioxidant potential have been encountered as efficient retardants of browning reactions.[11,23]

In the present work attempts have been made to inhibit PPO-mediated browning in apple juice by treatment with GSH (reduced form) and CA. Kinetic studies were carried out to understand the nature of the inhibition process.

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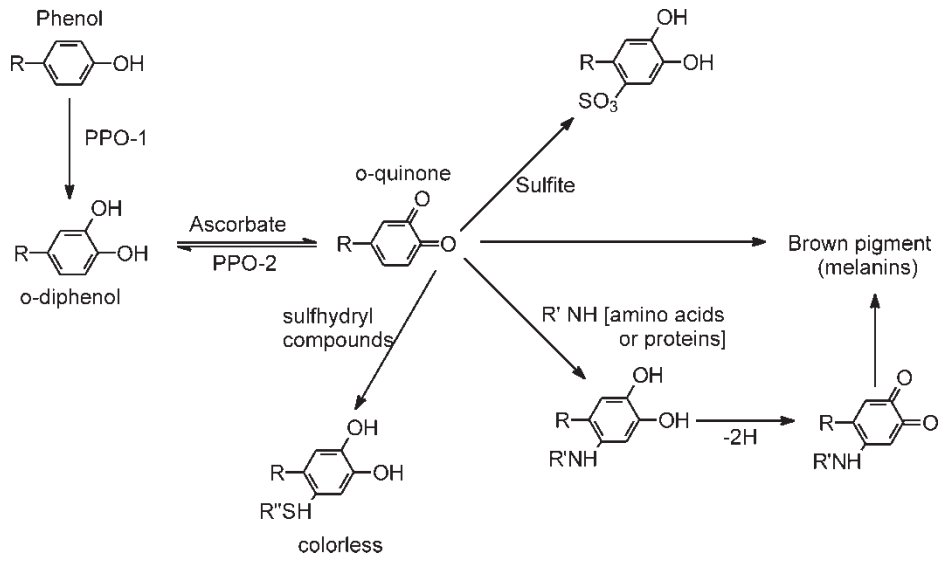

FIGURE 1 Reaction scheme and chemical approaches for controlling enzymatic browning (Labuza *et al.* 1992).

MATERIALS AND METHODS

Materials

Apples were purchased from the local market at Nanded city, GSH and L-DOPA (3,4-dihydroxy phenyl L-alanine) a substrate for PPO[12] were obtained from S. D. Fine Chemicals Ltd. Mumbai. CA was obtained from SISCO Chemical Industries Mumbai. Other chemicals were of AR grade.

Determination of Rate of Browning (Quinone Formation) Using GSH and CA

Apples (100 g) were chilled, peeled and blended. The preparation was diluted with 200 ml distilled water filtered through 8 layers of muslin cloth and re-filtered through filter paper to obtain a clear juice. Individual concentrations (1 to 5 mM) of GSH and CA were prepared from 5 mM stock solutions. Two sets of six containers (for GSH and CA) each containing 9 ml juice were used for studies. To these containers, 1 ml individual concentration of GSH and CA was added serially and the 6th container was kept as a control. At 10 min intervals, samples of the mixtures were examined spectrophotometrically at 420 nm (Figures 2–3).

Extraction, Protein Determination, Assay and Activity Measurements of PPO

A semi-pure preparation of PPO was used to provide information on the nature of PPO inhibition by GSH and CA. PPO extraction was carried out using 50% (w/v) saturated ammonium sulfate. In brief, 2 × 100 g freshly peeled apple pieces were blended and extracted in chilled citrate buffer (pH 4.8, 0.1 M) to a final volume of 50 ml, which was filtered through 8 layers of muslin cloth and centrifuged. To achieve 50% saturation with ammonium sulfate, 25 g of ammonium sulfate was added to 50 ml filtrate with continuous stirring. The mixture was kept at 0°C for 3 h for enzyme precipitation to occur. After precipitation the residue was collected by centrifugation

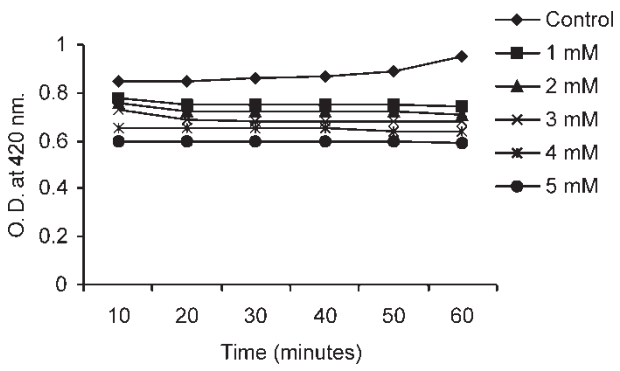

FIGURE 2 Rate of browning (quinone formation) in apple juice in control and Glutathione treated samples. The results presented are the mean values of duplicate measurements.

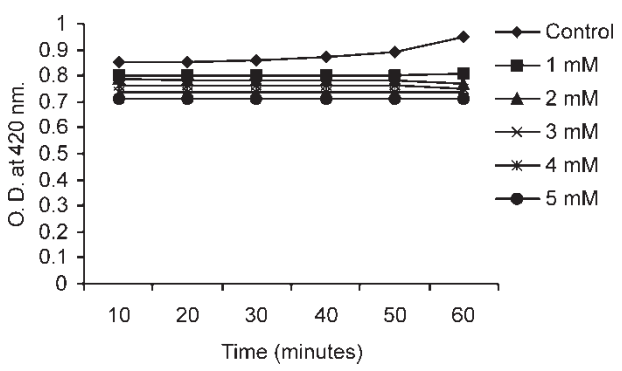

FIGURE 3 Rate of browning (quinone formation) in apple juice treated with different concentrations of cinnamic acid. The results presented are the mean values of duplicate measurements.

TABLE I Effect of different concentrations of GSH and CA on activity of PPO

	Activity (U/mg)	
Inhibitor (mM)	GSH	CA
0	438	438
1	384	411
2	274	385
3	268	356
4	265	328
5	262	326

The results presented are the mean values of duplicate measurements.

and re-dissolved in citrate buffer (pH 4.8, 0.1 M). The preparation was stored at 4°C for further use. Irrespective of the speed of the whole procedure the preparation of PPO always ended up with a slightly brown coloration.

The protein determination and PPO assay were carried out by the published methods.[13,14] The determination of PPO activity with individual concentrations (1–5 mM) of GSH and CA was carried out separately. The reaction mixture contained L-DOPA (1 ml, 2 mM), enzyme solution (0.5 ml, 1.35 mg protein), 1 ml individual concentration of either GSH or CA, and citrate buffer (pH 4.8, 0.1 M) to a final volume 3 ml. One unit of enzyme activity was defined as the amount of enzyme which caused an increase in absorbance of 0.001 min^{-1} at 470 nm at 25°C and pH 4.8 which corresponds to the formation of 0.01 μM of product (dopachrome). The profile of PPO activity in different concentrations of GSH and CA is shown in Table I.

Kinetics of Inhibition of PPO by GSH and CA

To understand the nature of the PPO inhibition, sets of five concentrations of GSH and CA (1–5 mM) were used and compared with the kinetic behavior of a control set (without addition of GSH or CA). For kinetic studies the enzyme concentration and total volume of reaction mixture was kept constant but the substrate concentration (L-DOPA) varied (2–10 mM). The results were plotted as double reciprocal plots (Figures 4–5).

RESULTS

The rate of the browning reaction in apple juice treated with different concentrations of GSH and CA was observed for one hour after preparation of the juice. Figures 2 and 3, shows the absorbance at 420 nm vs time for 1–5 mM concentrations of GSH and CA. The results summarized in Figures 2–3 clearly indicate that the individual treatment of adding GSH and CA reduces the rate of browning over time as compared to the control. The amount of protein from 100 g apples was estimated to be 2.7 mg/ml. The results tabulated in Table I show that with increase in time and concentration of GSH/CA, the process of inhibition increases. GSH is seen to be a more potent inhibitor of PPO than CA.

The kinetic results plotted as double reciprocal Lineweaver-Burk plots (Figures 4 and 5) for GSH and CA respectively show that the nature of the inhibition is non-competitive and competitive

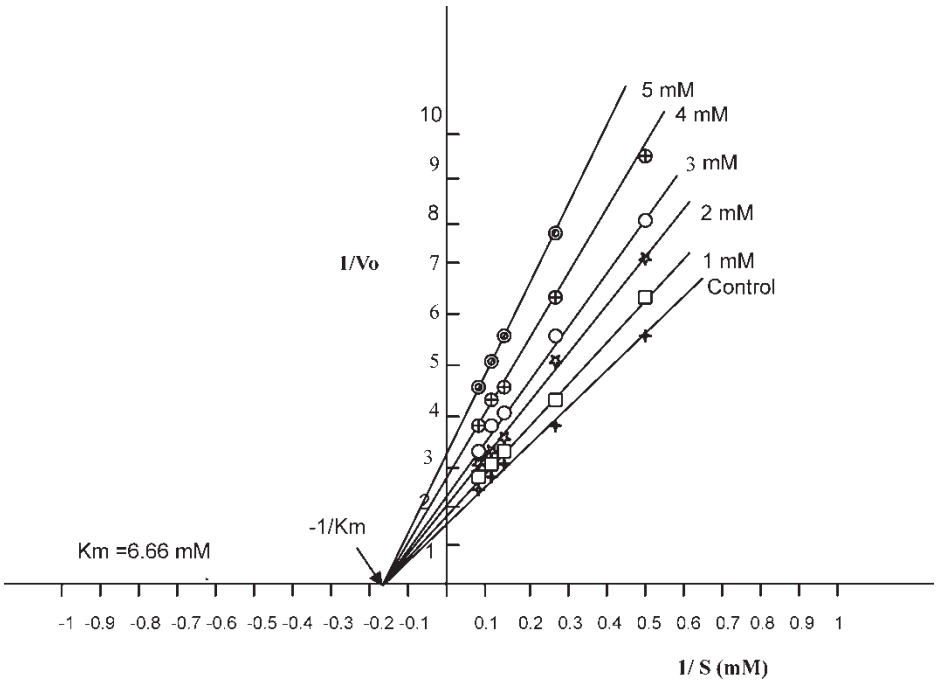

FIGURE 4 Double reciprocal Lineweaver-Burk plot of inhibition of PPO by glutathione (1–5 mM) with L-dopa (1 ml, 2–10 mM), enzyme (0.5 ml, 1.35 mg protein) in citrate buffer (0.1 M, pH 4.8) to a total volume of 3 ml.

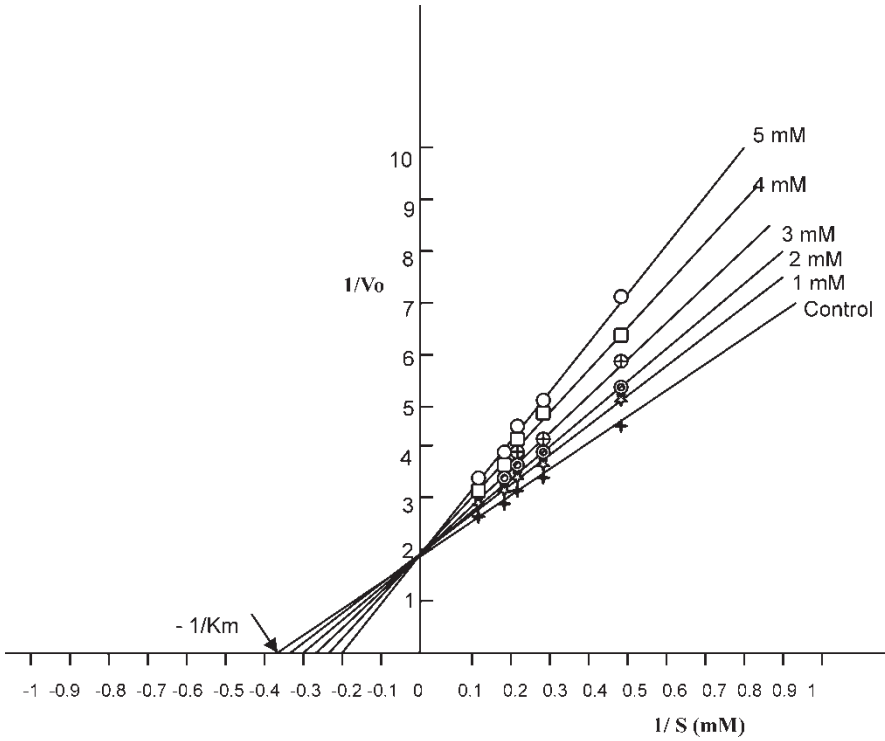

FIGURE 5 Double reciprocal Lineweaver-Burk plot of inhibition of PPO by cinnamic acid (1–5 mM) with L-Dopa (1 ml, 2–10 mM), enzyme (0.5 ml, 1.35 mg protein) in citrate buffer (0.1 M, pH 4.8) to a total volume of 3 ml.

respectively. The Km value (6.66 mM) calculated for GSH remains constant, while V_{max} reduces ($1/V_0$ increases). The Km values calculated for 1–5 mM concentrations of CA were found to be 3.12 (1.0 mM), 3.35 (2.0 mM), 3.84 (3.0 mM), 4.34 (4.0 mM) and 5.26 (5.0 mM) respectively, while the overall value of V_{max} remains constant as compared to V_{max} of GSH.

DISCUSSION

PPO inhibitors are broadly classified into two types. Type I inhibitors interact with the copper site in the enzyme while type II inhibitors interferes with the site for phenolic substances. Type I and II show a competitive and non-competitive mode of inhibition respectively.[15] The non-competitive type of inhibition is reversible and cannot be overcome by increasing substrate concentration. The inhibitor binds to the enzyme or enzyme substrate complex.[16] The competitive mode of inhibition can be reversed by increase in substrate concentration. The inhibitor binds only to the free enzyme.[17] Km values indicate affinity of an enzyme towards its substrate; the greater the value of Km, the less is the affinity.[18]

GSH (reduced form), is a tripeptide (γ-glu-cys-gly) having reducing properties with a broad redox potential.[19] Due to the redox active thiol group, GSH is reported to be an antioxidant.[20] The thiol-group (–SH) is present in the cysteine residue of GSH. According to Richard and Forget,[21] L-cysteine prevents the browning reaction by trapping *o*-quinone as cysteine–quinone addition compounds (CQAC). Here, GSH was shown to be a non-competitive inhibitor of PPO.

Phenolic acids are reported to be effective antioxidants and exhibit reduction potentials in the range of 540–700 mV.[22] Antioxidant properties of phenolic acids arise from their high reactivity as hydrogen or electron donors, and from their ability to chelate transition metal ions.[23] Free cinnamic acid and its esterified derivaties are also reported to be antioxidants towards copper- catalyzed LDL (low density lipoprotein) oxidation *in vitro*.[24] Copper is essential for PPO activity and complexation of copper situated at the active site of the enzyme results in enzyme inhibition.[25] The competitive mode of inhibition of PPO by cinnamic acid may be due to chelation of copper from the active site of PPO.

In conclusion it is clear from the present studies that GSH and CA are effective in delaying the browning reaction in apple juice by acting as inhibitors of PPO. GSH a biochemical having potent cellular reductant activity, is also involved in regulation of the cell cycle and acts as a defence compound against oxidative stress.[20] Phenolic acids, including CA, are found in all fruits and vegetables and in virtually all parts of the plant.[26] Free radicals (bi-products of normal metabolism) cause extensive damage to DNA, protein and lipid and act as major contributors to aging and degenerative diseases

of aging such as cancer, cardiovascular disease, immune system decline and cataract. The Antioxidant potential of phenolics efficiently inactivate various free radicals[27] and it is this reputation of phenolics that has driven their cocamination in research in different areas in recent years. Being dietary and nontoxic, GSH and CA addition to apple juice may be useful for improving health.

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